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CONTRACTING ORGANIZATION: Harvard College
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14. ABSTRACT
Purpose: The overarching goal of the grant was to characterize molecular heterogeneity in multifocal prostate cancer. Aim 1 focused on heterogeneity in PTEN loss in tumor tissue and prostate cancer prognosis. Aim 2 aimed to compare gene expression profiles between primary and lymph node metastases Scope: During the grant term, Dr. Batista received IRB approval, completed coursework to augment her expertise in prostate cancer epidemiology, coordinated meetings with collaborators, aided in specimen and data collection for the proposed work, lead the statistical analyses, and published the findings in peer-reviewed journals. Major Findings: PTEN loss was heterogeneous in prostate cancer foci, and was predictive of disease relapse. In related manuscripts that Dr. Batista co-authored and published, tumor expression of PSMA and genetic mutations in the SOD2 gene were associated with prostate cancer progression. Dr. Batista also published a review article on AMPK activation in cancer in Molecular Cancer Research. Significance: The clinical significance of the project was to better characterize putative prognostic markers for prostate cancer.

15. SUBJECT TERMS

None Listed

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INTRODUCTION

Since approximately 1 in 8 men with prostate cancer in the US will die of their disease, it is critical to identify early in the disease course those men who are likely to progress in order to administer appropriate therapies. Several tumor-derived RNA expression signatures have been developed to improve upon the prognostic value of known clinical parameters (e.g. Gleason score, tumor stage, PSA levels) to predict prostate cancer recurrence or death. However, hundreds of genes have been identified in the current signatures, and it is unclear which ones are biologically relevant for metastatic spread due, in part, to the difficulty in obtaining metastatic specimens and inherent tumor heterogeneity. The current final report focuses on tumor heterogeneity of one of the four genes: Phosphatase and tensin homolog (PTEN). PTEN is a well-known tumor suppressor gene that acts as a negative regulator of the PI3K/AKT pathway. Loss of PTEN expression has been associated with aggressive prostate cancer and adverse outcomes in several studies.[2,3] Since PTEN expression appears to have prognostic utility and may guide treatment decisions, it is important to characterize heterogeneity as prostate biopsies sample only a portion of the existing tumor. However, detailed characterization of PTEN heterogeneity within and between tumor foci in prostate cancer patients is limited. To our knowledge, only one prior study has assessed the distribution of PTEN loss in multifocal prostate cancer: among 142 patients that underwent radical prostatectomy, PTEN deletion was observed in 42% of patients and was significantly correlated with higher tumor Gleason grade. [4] Our study is slightly larger and assesses PTEN protein loss using an alternative, valid method of immunohistochemistry.[5,6] Furthermore, we were able to link PTEN loss with long-term clinical outcomes.

KEY WORDS

Prostate cancer, tumor heterogeneity, phosphatase and tensin homolog (PTEN), prognosis

OVERALL PROJECT SUMMARY

Task 1. Aim 1: Characterize heterogeneity of a 4-gene signature across prostate tumor nodules and validate its prognostic potential

IRB approval was obtained for this project at Harvard School of Public Health in October 2012. The tissue microarray of approximately 200 prostate cancer patients that underwent radical prostatectomy, including approximately one third with multi-focal disease, was constructed in 2013. Since the original grant proposal, the funding source to measure the 4-gene signature in these tumor specimens became unavailable to perform the assay.[1] In year 2, Dr. Batista secured an alternative source to measure one of the genes (PTEN) in the laboratory of Dr. Tamara Lotan at Johns Hopkins School of Medicine. Dr. Lotan is an expert pathologist and has developed an immunohistochemical method for measuring PTEN expression that is valid and methodologically easier than the alternative FISH assay.[5,6] The following are our current

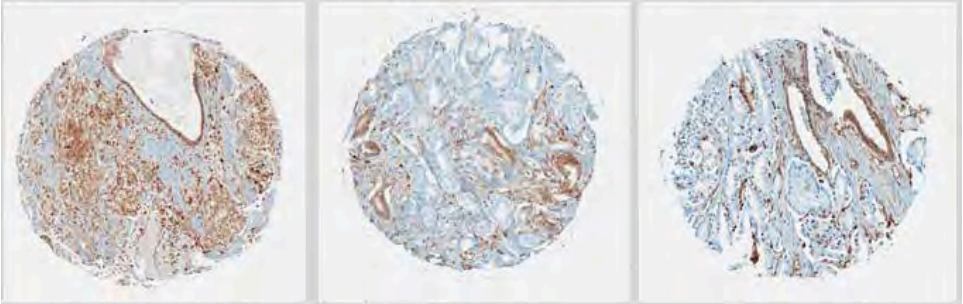
findings on intratumoral heterogeneity of PTEN staining in a Swedish cohort of prostate cancer patients.

PTEN was evaluated in archival tumor tissue from 198 prostate cancer patients diagnosed from 1989-2005 (**Table 1**). A single tumor focus was evaluated for PTEN protein expression in 70% of patients, while 2-4 tumor foci were evaluated in 30% of patients. PTEN loss was assigned if the patient had any areas of the tumor showing markedly decreased or completely negative immunohistochemical staining (at least 10% of cells), as compared with benign epithelium and stromal cells within the tumor. A patient was scored as **homogeneous PTEN expression** if PTEN was expressed in all cores of all foci; **homogeneous PTEN loss** if at least one tumor focus had uniform loss in all cores; and **heterogeneous PTEN loss** if one or more tumor foci had non-uniform loss (**Figure 1**).

Table 1: Select characteristics of 198 prostate cancer patients from Sweden

Year of diagnosis	1989-2005
Age at diagnosis	45-74 yrs
Average follow-up time	10.7 yrs
Gleason score	
≤ 6	42 (21%)
7	127 (64%)
≥ 8	29 (15%)
N tumor foci on TMA	
1	139 (70%)
2	47 (24%)
3-4	12 (6%)

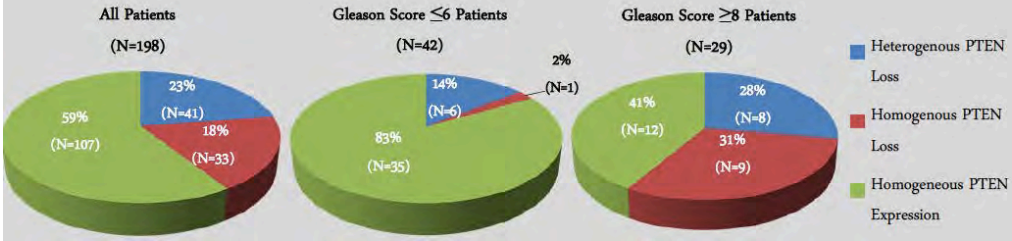
Fig.1: Representative Images of PTEN Protein Expression



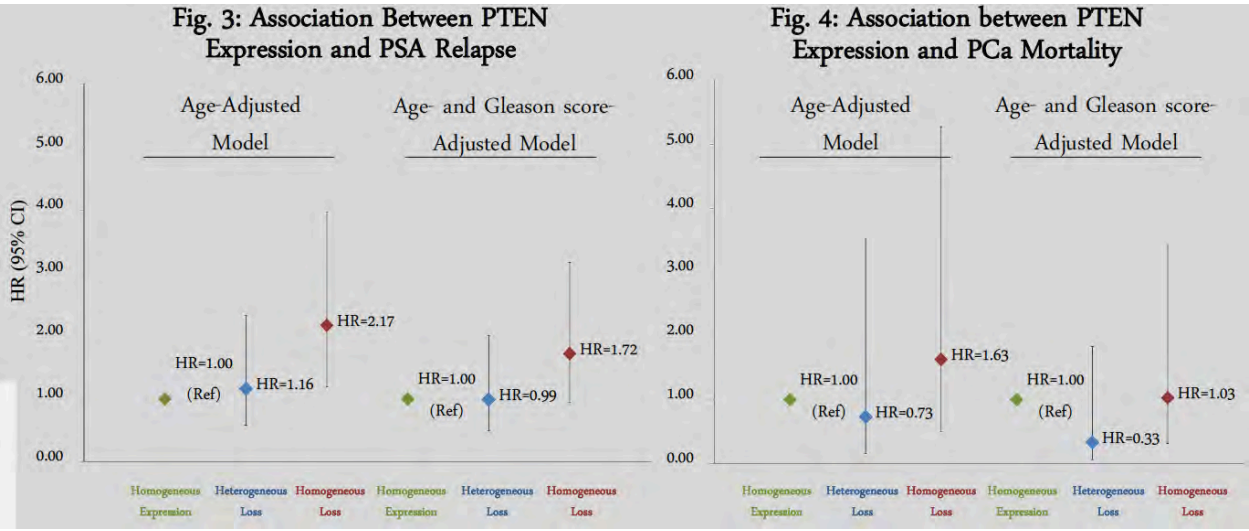
Homogeneous PTEN Expression | Heterogeneous PTEN Expression | Homogenous PTEN Loss

We found that homogenous PTEN loss was present in 18% of all patients, and that heterogeneous PTEN loss was present in 23% of patients (**Figure 2**). This is in agreement with Yoshimoto et al. who found that PTEN deletion was present in 42% of prostate cancer patients,[4] whereas Gumuskaya et al. noted PTEN loss in 53% of patients.[6] In our study, the distribution of PTEN loss differed by Gleason score, where only 2% of Gleason score ≤6 patients had homogeneous PTEN loss, compared to 31% among Gleason score ≥8 patients.

Fig. 2: Distribution of PTEN Expression



We used Cox proportional hazards regression to calculate multivariable hazard ratios and 95% confidence intervals for the association between PTEN loss with PSA relapse (n=57 events) and prostate cancer-specific mortality (n=14 events). Homogenous PTEN loss was associated with a statistically significant 2.17-fold increased risk of PSA relapse (**Figure 3**) and a non-significant 1.63-fold increased risk of prostate cancer-specific mortality (**Figure 4**) in age-adjusted models. After adjusting for Gleason score, the associations were attenuated. Heterogeneous PTEN loss was not associated with PSA relapse or prostate cancer-specific mortality. Our findings are in agreement with two prior studies that found PTEN loss to be associated with time to prostate cancer metastasis and death.[2,5] We are currently drafting a manuscript for submission to a peer-reviewed journal.



Task 2. Aim 2: Identify genes critical for metastatic progression to lymph nodes in prostate cancer

IRB approval was obtained at Harvard School of Public Health in October 2012. Our collaborator, Dr. Ove Andren, finished the tissue collection of within-person primary and lymph node-positive archival tumor specimens in Sweden. Of the hundreds of records reviewed, 5 patient-matched radical prostatectomy and positive lymph node samples were identified. This is less than the expected number of 10-15 matched pairs. However, Dr. Andren was able to identify an addition 50 patients with positive lymph nodes for which the diagnostic biopsy specimen is available for analysis. Since biopsy specimens have very small amounts of tumor tissue, and thus low yields of mRNA, we are actively devising a feasible plan to best address these methodological challenges for mRNA expression profiling. Thus, we have not been able to generate results for this particular aim as of yet.

Despite these challenges, Dr. Batista has made excellent progress on 3 related projects that explore key biomarkers in prostate cancer prognosis. First, in 2013, Dr. Batista and co-authors published a manuscript on prostate specific membrane antigen (PSMA) and prostate cancer-specific mortality in *Cancer Epidemiology, Biomarkers, and Prevention*.[7] In *Clinical Genitourinary Cancer* in2015, Dr. Batista was co-author on a manuscript that found an

association between common mutations in the superoxide dismutase-2 (SOD2) gene and prostate cancer recurrence after radiation for prostate cancer in a low-risk subset of patients.[8] Finally, Drs. Batista, Zadra, and Loda were invited to write a review article in *Molecular Cancer Research* on the role of AMPK activation in cancer.[9] All three of these manuscripts are included in the appendices of this final report.

Task 3. Mentored training with Dr. Mucci

Drs. Mucci and Batista have completed this task by meeting regularly to discuss progress on the specific aims of the project, as well as evaluating short- and long-term goals.

Task 4. Coursework

In year 1, Dr. Batista took several courses in pathology, molecular epidemiology, and biostatistics. In September 2012, Dr. Batista attended a 2-hr course on "Introduction to Microarrays and Affymetrix Data analysis using R/Bioconductor" at Harvard Medical School where she familiarized herself with the R programming language. In October 2013, Dr. Batista attended a 2-hr course on "Whole Transcript Expression analysis using Gene and Exon 1.0 ST arrays" at Harvard Medical School. The course further developed her knowledge of the R programming language and techniques for analyzing expression array data. In January 2013, Dr. Batista completed EPI508 (Pathology for Epidemiologists; 1-week course) with a grade of 'Pass' at Harvard School of Public Health. The objective of the course was to provide an overview of tumor classification systems, histology, immunohistochemistry, and other molecular techniques used in epidemiologic research involving tumor specimens. From January-May 2013, Dr. Batista completed BIO508 (Genomic Data Manipulation; semester-long course) at Harvard School of Public Health with a grade of "A." The course taught computational methods for genomic data analysis using the Python programming language and online, publically available research tools. All formal coursework was completed in year 1.

In year 2, Dr. Batista continued her training by attending an "Introduction to Network Medicine" course (October 2013) hosted by the Harvard Catalyst. The 3-day course provided an introduction to the identification and investigation of molecular networks that underlie disease etiology and treatment.

Task 5. Meetings and seminars

Dr. Batista has attended numerous meetings and seminars as planned in years 1 and 2. She has attended two bi-weekly meetings, including a prostate cancer epidemiology meeting and pathology-epidemiology working group. Monthly meetings that Dr. Batista attends include meetings for the Prostate Cancer SPORE at Dana-Farber/Harvard Cancer Center and for prostate cancer journal club at Harvard School of Public Health. Dr. Batista also took part in a special week-long workshop entitled "Integrative Molecular Epidemiology Workshop" in July 2013 in Boston, MA, sponsored by the American Association of Cancer Research. This workshop addressed the challenges faced when integrating high-dimensional data from multiple sources

in order to inform disease etiology and outcomes. In March 2015, Dr. Batista presented an abstract on PTEN loss in multifocal prostate cancer at the Multi-Institutional Prostate Cancer Program Retreat in Ft. Lauderdale, Florida.

KEY RESEARCH ACCOMPLISHMENTS

- Publication of four co-authored manuscripts in peer-reviewed journals [7-9]
- Literature review of current studies comparing molecular differences in metastatic versus primary prostate cancer
- Completion of a tissue microarray with prostate tumor specimens representing patients with multi-focal disease
- Completion of statistical analysis and initial manuscript preparation for Aim 1
- Development of a prostate tumor tissue resource that utilizes patient-matched primary and lymph node-positive prostate cancer specimens

CONCLUSION

Dr. Batista made significant progress during this Career Development Award through coursework, teaching, developing tumor tissue shared resources, attending research conferences, and publishing manuscripts in peer-reviewed journals. Regarding career accomplishments, Dr. Batista was promoted to Instructor in the Department of Medicine at Harvard Medical School/ Brigham and Women's Hospital in July 2013. Dr. Batista has worked to overcome the challenge of finding an alternative means of performing the assays for Aim 1, and has helped develop the tissue resource for Aim 2. The current findings on PTEN loss in multifocal prostate cancer, detailed in the Overall Project Summary section of this report, highlight that PTEN loss is a common event in prostate cancer, and often is characterized by heterogeneous expression across tumor foci. This information is clinically relevant when evaluating PTEN as a potential prognostic marker in prostate cancer patients. In summary, Dr. Batista, through her work on this Career Development Award, has made important contributions to the understanding and characterization of molecular and prognostic heterogeneity in prostate cancer.

PUBLICATIONS, ABSTRACTS, AND PRESENTATIONS

Note. Dr. Batista has published under the last names **Kasperzyk** and **Batista**.

1. Lay press
    a. Nothing to report.
2. Peer-reviewed scientific journals
    a. **Kasperzyk JL**, Finn SP, Flavin R, Fiorentino M, Lis R, Hendrickson WK, Clinton SK, Sesso HD, Giovannucci EL, Stampfer MJ, Loda M, Mucci LA. Prostate-specific membrane antigen protein expression in tumor tissue and risk of lethal prostate

cancer. *Cancer Epidemiol Biomarkers Prev* 2013;22:2354-63. doi: 10.1158/1055-9965.EPI-13-0668.

b. Margalit DN, Jordahl KM, Werner L, Wang X, Gwo-Shu Lee M, Penney KL, **Batista JL**, Martin NE, Chan JM, Kantoff PW, Stampfer MJ, Nguyen PL, Mucci LA. Germline variation in superoxide dismutase-2 (SOD2) and survival outcomes after radiation therapy for prostate cancer: results of a test and validation set analysis. *Clin Genitourin Cancer* 2015 Jan 3. pii: S1558-7673(14)00292-4. doi: 10.1016/j.clgc.2014.12.018. [Epub ahead of print]

3. Invited articles
    a. Zadra G, **Batista JL**, Loda M. Dissecting the Dual Role of AMPK in Cancer: from Experimental to Human Studies. *Mol Cancer Res*. 2015 May 8. pii: molcanres.0068.2015. [Epub ahead of print]
4. Abstracts
    a. **Batista JL**, Lotan TL, Morais CE, Carlsson J, Svensson MA, Mucci LA, Andrén O, Loda M. 'PTEN loss in multifocal prostate cancer.' Multi-Institutional Prostate Cancer Program Retreat in Ft. Lauderdale, Florida; March 2015.
5. Presentations
    a. Invited speaker for 'Prostate Cancer Epidemiology' lecture at Boston University School of Public Health; October 2013, April 2014, and April 2015

INVENTIONS, PATENTS AND LICENSES

Nothing to report.

REPORTABLE OUTCOMES

- Publication of three manuscripts in peer-reviewed journals from 2013-2015 [7-9]

OTHER ACHIEVEMENTS

- Completion of a tissue resource by colleague (Dr. Ove Andren) that utilizes tissue microarray technology to catalog >200 prostate cancer patients with single and multi-focal prostate tumor specimens. These tissue microarrays were used for the analyses in Aim 1, and are available as a resource for any of our collaborators who wish to study protein expression and histological differences across tumor foci in this patient population.
- Development of a tissue resource that combines within-person primary and lymph node-positive prostate cancer specimens. This resource is coordinated in Sweden by Dr. Ove Andren and the archival tumor specimens are readily available for research purposes (Aim 2).
- Became co-investigator on funded R01 project (PI: Massimo Loda, Dana-Farber Cancer Institute) entitled 'Molecular link between metabolic syndrome and prostate cancer.'

- Became co-investigator on funded Dana-Farber Cancer Institute, A. David Mazzone Disparity Research Award (PI: Mark Preston, Brigham and Women's Hospital) entitled 'Do baseline prostate specific antigen (PSA) levels predict advanced prostate cancer in African American men?'
- Applied for Prevent Cancer Foundation award (PI: Julie Batista, Brigham and Women's Hospital) in August 2014 entitled 'Dairy intake in adolescence/adulthood and advanced prostate cancer risk.'
- Applied for Harvard Catalyst KL2/Catalyst Medical Research Investigator Training Program in May 2014 entitled 'Tumor biomarkers, quality of life, and long-term outcomes among prostate cancer patients.'
- Applied for American Institute for Cancer Research award in May 2014 entitled 'Healthy lifestyle to prevent lethal prostate cancer: exploration of underlying mechanisms.'

OPPORTUNITIES FOR TRAINING AND PROFESSIONAL DEVELOPMENT

During the grant period, Dr. Batista was able to take a number of graduate-level courses at the Harvard School of Public Health and Harvard Medical School as outlined in Task 4 of the 'Overall Project Summary' section above. She also attended a 3-day seminar on Network Medicine hosted by the Harvard Catalyst and a week-long workshop on Integrative Molecular Epidemiology hosted by the American Association for Cancer Research. These two workshops, along with attendance at national conferences, provided important opportunities for networking and professional growth.

In the past 2 years, Dr. Batista helped write two grants in which she became co-investigator: NIH/NCI R01CA131945 (PI: Massimo Loda) entitled "Molecular link between metabolic syndrome and prostate cancer" and a Dana-Farber Cancer Institute, A. David Mazzone Disparity Research Award (PI: Mark Preston) entitled "Do baseline prostate specific antigen (PSA) levels predict advanced prostate cancer in African American men?" She also applied for numerous grants as co-investigator and PI to various grant mechanisms offered by the Department of Defense, NIH, Dana-Farber Cancer Institute, Harvard Catalyst, American Institute for Cancer Research, and Prevent Cancer Foundation.

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8. Margalit DN, Jordahl KM, Werner L, Wang X, Gwo-Shu Lee M, Penney KL, Batista JL, Martin NE, Chan JM, Kantoff PW, Stampfer MJ, Nguyen PL, Mucci LA. Germline variation in superoxide dismutase-2 (SOD2) and survival outcomes after radiation therapy for prostate cancer: results of a test and validation set analysis. *Clin Genitourin Cancer* 2015 Jan 3. pii: S1558-7673(14)00292-4. doi: 10.1016/j.clgc.2014.12.018. [Epub ahead of print]
9. Zadra G, Batista JL, Loda M. Dissecting the Dual Role of AMPK in Cancer: from Experimental to Human Studies. *Mol Cancer Res*. 2015 May 8. pii: molcanres.0068.2015. [Epub ahead of print]

APPENDICES

8th ANNUAL MULTI-INSTITUTIONAL PROSTATE CANCER PROGRAM RETREAT

March 15-17, 2015
W Hotel Fort Lauderdale
Fort Lauderdale, Florida

ABSTRACT SUBMISSION FORM
Due date: November 21, 2014

Please Note: These abstracts are to be submitted for posters with the knowledge that some may be chosen for a talk or poster presentation.

First Name: Julie

Last Name: Batista

Check one of the following boxes:

Organization:
- [x] Dana-Farber Harvard Cancer Center
- [ ] Fred Hutchinson Cancer Research Center
- [ ] Johns Hopkins Medical Institutions
- [ ] Memorial Sloan-Kettering Cancer Center
- [ ] University of Michigan Comprehensive Cancer Center

PI of Your Research Program/Lab: Massimo Loda

Telephone: 617-525-2242

Email: hpjlk@channing.harvard.edu

ABSTRACT
Your complete abstract should be no longer than 3,000 characters (450–500 words).
Insert text following the colon after each heading

Title: PTEN loss in multifocal prostate cancer

Complete Author Listing:

Julie L. Batista, ScD[1,2], Tamara L. Lotan, MD[3], Carlos E. Morais, MD[3], Jessica Carlsson, PhD[4], Maria A. Svensson PhD[4], Lorelei A. Mucci, ScD[1,2], Ove Andrén, MD, PhD[4], Massimo Loda, MD[5]

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[2] Department of Epidemiology, Harvard School of Public Health, Boston, MA

[3] Department of Pathology, Johns Hopkins University School of Medicine, Baltimore, MD

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[5] Center for Molecular Oncologic Pathology, Dana-Farber Cancer Institute, Boston, MA

Body:

Background. Phosphatase and tensin homolog (PTEN) is a well-known tumor suppressor gene that acts as a negative regulator of the PI3K/AKT pathway. Loss of PTEN expression in prostate tumors has been associated with higher Gleason grade, higher tumor stage, and adverse outcomes in several patient populations. However, studies with detailed characterization of heterogeneity in PTEN loss within and between tumor foci are limited. **Methods.** The study population was 198 surgically treated prostate cancer patients diagnosed from 1989-2005 at Örebro University Hospital, Sweden. Tissue microarrays were constructed from whole-mount prostatectomy specimens with three 0.6-mm tumor cores sampled per focus. Of the 198 patients evaluated for PTEN protein expression, 59 (30%) had 2-4 tumor foci. PTEN loss was assigned if the tumor core had any areas showing markedly decreased or completely negative immunohistochemical staining (at least 5% of cells), as compared with benign epithelium and stromal cells within the tumor. We assigned patients as homogeneous PTEN expression (PTEN expressed in all cores of all foci), homogeneous PTEN loss (at least one focus with uniform loss in all cores), or heterogeneous PTEN loss (one or more foci with non-uniform loss). Cox proportional hazards regression was used to calculate multivariable hazard ratios (HR) and 95% confidence intervals (CI) for the association of PTEN loss with PSA relapse (n=57) and prostate cancer-specific mortality (n=14). **Results.** PTEN loss was detected in 37% of patients: n=41 with heterogeneous and n=33 with homogeneous loss. PTEN loss was more likely to be detected in patients with multifocal disease (53% with PTEN loss) versus patients with a single focus (31% with PTEN loss). PTEN status differed across foci in 44% of the 59 men with multifocal disease; the PTEN loss occurred in the higher Gleason score focus in 75% of the 16 patients with disparate Gleason scores across foci. Among all patients, PTEN loss was significantly correlated ($p<0.001$) with higher Gleason score: 57% of Gleason score $\geq$8 foci versus 11% of Gleason score $\leq$6 foci had either homogeneous or heterogeneous PTEN loss. Compared to patients with homogeneous PTEN expression, homogeneous PTEN loss was associated with a significantly increased risk of PSA relapse (HR=2.17; 95% CI: 1.19, 3.97) and a non-significant increase in prostate cancer-specific mortality (HR=1.63; 95% CI: 0.50, 5.28), adjusting for age at diagnosis. After additionally adjusting for Gleason score, the associations with homogenous PTEN loss were attenuated: HR=1.72 (95% CI: 0.94, 3.17) for PSA relapse and HR=1.03 (95% CI: 0.31, 3.42) for prostate cancer-specific mortality. In contrast, heterogeneous loss of PTEN was not significantly associated with PSA relapse (HR=1.16; 95% CI: 0.58, 2.33) or prostate cancer-specific mortality (HR=0.73; 95% CI: 0.15, 3.53), adjusting for age at diagnosis. **Conclusion.** Heterogeneity in PTEN loss either within or across tumor foci is common among prostate cancer patients. Our findings suggest that homogeneous versus heterogeneous PTEN loss should be considered when evaluating PTEN as a potential prognostic marker.

Acknowledgments/Funding: Department of Defense Prostate Cancer Research Program (W81XWH-12-1-0072), Dana-Farber Cancer Institute Mazzone Awards Program (2012_CD_171), and NIH/NCI (R01 CA131945)

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Cancer Epidemiology, Biomarkers & Prevention

Prostate-Specific Membrane Antigen Protein Expression in Tumor Tissue and Risk of Lethal Prostate Cancer

Julie L. Kasperzyk, Stephen P. Finn, Richard Flavin, et al.

Cancer Epidemiol Biomarkers Prev 2013;22:2354-2363. Published OnlineFirst October 15, 2013.

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Cancer Epidemiology, Biomarkers & Prevention

Research Article

Prostate-Specific Membrane Antigen Protein Expression in Tumor Tissue and Risk of Lethal Prostate Cancer

Julie L. Kasperzyk[1,3], Stephen P. Finn[6,7], Richard Flavin[6,7], Michelangelo Fiorentino[1,6,8], Rosina Lis[6], Whitney K. Hendrickson[1,3], Steven K. Clinton[9], Howard D. Sesso[4], Edward L. Giovannucci[1,2,3], Meir J. Stampfer[1,2,3], Massimo Loda[5,6], and Lorelei A. Mucci[1,3]

Abstract

Background: Overexpression of prostate-specific membrane antigen (PSMA) in tumor tissue and serum has been linked to increased risk of biochemical recurrence in surgically treated prostate cancer patients, but none of the studies have assessed its association with disease-specific mortality.

Methods: We examined whether high PSMA protein expression in prostate tumor tissue was associated with lethal disease, and with tumor biomarkers of progression, among participants of two U.S.-based cohorts ($n = 902$, diagnosed 1983–2004). We used Cox proportional hazards regression to calculate multivariable HRs and 95% confidence intervals (CI) of lethal prostate cancer, defined as disease-specific death or development of distant metastases ($n = 95$). Partial Spearman rank correlation coefficients were used to correlate PSMA with tumor biomarkers.

Results: During an average 13 years of follow-up, higher PSMA expression at prostatectomy was significantly associated with lethal prostate cancer (age-adjusted $HR_{Quartile(Q)4vs.Q1} = 2.42$; $P_{trend} < 0.01$). This association was attenuated and nonsignificant (multivariable-adjusted $HR_{Q4vs.Q1} = 1.01$; $P_{trend} = 0.52$) after further adjusting for Gleason score and prostate-specific antigen (PSA) at diagnosis. High PSMA expression was significantly ($P < 0.05$) correlated with higher Gleason score and PSA at diagnosis, increased tumor angiogenesis, lower vitamin D receptor and androgen receptor expression, and absence of ets-related gene (*ERG*) expression.

Conclusions: High tumor PSMA expression was not an independent predictor of lethal prostate cancer in the current study. PSMA expression likely captures, in part, malignant features of Gleason grade and tumor angiogenesis.

Impact: PSMA is not a strong candidate biomarker for predicting prostate cancer–specific mortality in surgically treated patients. *Cancer Epidemiol Biomarkers Prev; 22(12); 2354–63. ©2013 AACR.*

Introduction

Prostate-specific membrane antigen (PSMA) is a type II transmembrane glycoprotein that is highly expressed in the normal prostate epithelium, and to a lesser extent in other tissues such as brain, liver, and kidney (1, 2). PSMA expression is higher in primary prostate tumors and metastatic lesions compared with benign tissue, and is positively associated with tumor grade and stage (3–7). Because of its high expression in malignant prostate tissue, PSMA has been used in immunoscintigraphy to monitor metastatic disease and as a target antigen for immunotherapy (8, 9).

PSMA may also have prognostic utility. Three studies of surgically treated prostate cancer patients showed that high PSMA protein expression in tumor tissue was associated with biochemical recurrence (5–7). Two of these studies found that PSMA overexpression was predictive of biochemical recurrence after multivariable adjustment for clinical parameters, such as tumor stage, grade, and preoperative prostate-specific antigen (PSA) levels (5, 6). However, Minner and colleagues did not find PSMA to be an independent predictor after adjusting for clinicopathologic features (7). High PSMA mRNA expression in preoperative peripheral blood cells, possibly detecting micrometastatic disease, similarly showed a positive association with biochemical

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recurrence in four prospective studies (10–13), a relationship not observed in the fifth study (14). No studies to date have investigated PSMA expression in relation to prostate cancer–specific mortality.

PSMA functions as a peptidase with both N-acetylated α-linked acidic peptidase and folate hydrolase activity (15, 16). *In vitro* and *in vivo* experiments have shown that high PSMA expression activates signaling pathways that promote tumor cell survival and proliferation (17). The association of PSMA with anaphase-promoting complex disrupts cell-cycle checkpoints, induces chromosomal instability, and contributes to aneuploidy (18). In addition, PSMA is negatively regulated by 1α,25-dihydroxyvitamin D_3 (19), a nutrient associated with reduced proliferation in animal models and prostate cancer cell lines (20, 21). Interestingly, androgen deprivation enhances PSMA expression (1, 22), and a role in the development of castration resistance has been hypothesized. Androgens stimulate *TMPRSS2:ERG* expression, a gene fusion mutation common in human prostate cancer (23), as the TMPRSS2 promoter has an androgen-responsive element, thus providing a potential link between inhibition of PSMA by androgen and ets-related gene (*ERG*) expression in fusion-positive prostate cancer cells (24). PSMA has also been identified as a regulator of new blood vessel formation (i.e., angiogenesis) in mouse models (25, 26). Although virtually absent from nonprostatic normal tissues, PSMA is expressed in the neovasculature of many solid tumors, thus underscoring its importance in tumor angiogenesis (27–30).

In this prospective study, our main objective was to determine whether tumor PSMA protein expression from primarily radical prostatectomy specimens was an independent predictor of prostate cancer–specific mortality in 902 participants of the Physicians' Health Study (PHS) and Health Professionals Follow-Up Study (HPFS). To identify potential mechanisms of PSMA in disease progression, we also evaluated correlations between PSMA expression and measures of cell proliferation, apoptosis, angiogenesis, and protein expression of vitamin D receptor (VDR), androgen receptor (AR), and ERG in prostate tumor tissue.

Materials and Methods

Study population

This study population of patients with prostate cancer is drawn from participants of the prospective PHS and HPFS studies for whom archival prostate tumor tissue, primarily from radical prostatectomy, was available for biomarker analysis. PHS I and II were randomized, placebo-controlled, double-blind trials for the prevention of cardiovascular disease and cancer. PHS I began in 1982 and evaluated aspirin and β-carotene among 22,071 U.S. male physicians (31); in 1997 PHS II randomized 7,641 physicians from PHS I and 7,000 new physicians to β-carotene, vitamin E, vitamin C, and multivitamins (32). All arms of the PHS I and II have been terminated (33–35), and the PHS continues to be followed annually. The HPFS began in 1986 with 51,529 U.S. male health care professionals (dentists, veterinarians, pharmacists, optometrists, osteopathic physicians, and podiatrists) who are prospectively followed on biennial questionnaires to collect lifestyle and medical information (36). This study was approved by the Partners Healthcare and Harvard School of Public Health Institutional Review Boards.

Clinical data and prostate cancer outcomes

Self-reported, incident cases of prostate cancer arising in the PHS (1983–2004) and HPFS (1986–2001) were confirmed by medical record and pathology report review by study investigators. In rare cases, prostate cancer diagnoses were identified on death certificates and confirmed by medical record, pathology report, and death certificate review. To ascertain clinical characteristics and disease-specific treatments or outcomes, information on tumor stage, PSA at diagnosis, body mass index (BMI), and metastases events during follow-up was collected from medical record and pathology report review, and from questionnaires sent to prostate cancer survivors (2004 onward). Pathologic tumor stage was available for 90% of patients, whereas the remaining had clinical stage information ($n = 89$) or were missing ($n = 2$). More than 97% of tumor specimens were re-reviewed by a study pathologist (M. Fiorentino and R. Flavin) to achieve uniformity of scoring, and the remaining were assigned clinical Gleason score. Cause of death was assigned via review of medical records and death certificates for the vast majority of participants, and secondarily via information from family. We defined lethal disease as death from prostate cancer or distant metastases (to bone or other organs, excluding lymph nodes) during follow-up. A total of 95 lethal events occurred: 29 in PHS and 66 in HPFS. We analyzed a composite of biochemical recurrence and lethal prostate cancer ($n = 231$) as a secondary endpoint, using the first recorded event as the event date. Biochemical recurrence was defined as PSA above 0.2 ng/mL after surgery sustained over two measures (when abstracted from medical records), or a report of biochemical recurrence by the participant or treating physician.

Tumor biomarker measurements

Tissue microarray construction. Formalin-fixed, paraffin-embedded archival tumor tissue specimens were obtained from the hospital pathology departments; 95% were from radical prostatectomy procedures and the remaining were from the transurethral resection of the prostate (TURP). Our pathologist reviewed all available slides to provide standardized Gleason grading and for identification of the areas of tumor tissue for tissue microarray construction blinded to outcome status (37). For this project, we used nine tissue microarrays constructed from areas of the dominant tumor nodule or highest Gleason grade, with at least three tumor cores (0.6 mm) sampled from each patient.

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PSMA immunohistochemistry. Protein expression of PSMA was ascertained on 5 μm sections of the tissue microarrays (pathologist: S.P. Finn). Antigen retrieval was by microwave in citrate buffer (3 × 5 minutes). We used a primary mouse monoclonal antibody (Clone E36, M3620; Dako) with 1:100 dilution for 60 minutes after treatment with a peroxidase block (Dako). An anti-mouse secondary antibody was applied, followed by a counterstain with hematoxylin (Sigma-Aldrich). PSMA expression was quantified using the Ariol platform (Genetix Corp.), a semiautomated, quantitative image analysis system, and defined as staining intensity (scale, 0–255) multiplied by percentage area staining positive (scale, 0%–100%) for a given tumor field on each tissue microarray core. All nine microarrays were stained in the same batch, and positive and negative controls were included according to the antibody manufacturer's instructions.

Proliferation and apoptosis indices. Cellular proliferation was assessed on 5 μm sections of the tissue microarrays using rabbit polyclonal anti-Ki67 antibody (Vector Laboratories), diluted at 1:2,000 with citrate-based antigen retrieval solution (pathologist: S.P. Finn). Ki67 staining was visualized using the Ariol platform (Genetix Corp.), and quantified as the percentage of positively stained nuclei in the tumor region of each core. Apoptosis was evaluated on 5 μm sections of the tissue microarrays using the ApopTag Peroxidase *In Situ* Kit (Chemicon International) according to the manufacturer's instructions, and defined as the percentage of tumor cells undergoing apoptosis (pathologist: M. Fiorentino; ref. 38).

VDR, AR, and ERG immunohistochemistry. VDR expression was calculated on 5 μm sections of the tissue microarrays using rabbit polyclonal anti-VDR antibody (Santa Cruz Biotechnology) at a dilution of 1:600 as previously described (pathologist: R. Flavin; ref. 37). VDR expression was quantified as a combination of percentage area that was positively stained and staining intensity using CRi Vectra, a semiautomated, quantitative image analysis system (CRi). AR expression was calculated on 5 μm sections of the tissue microarrays using rabbit polyclonal anti-AR antibody (Upstate/Millipore) at a dilution of 1:100 (pathologist: S.P. Finn). Mean intensity (scale, 0–255) of AR staining in the nucleus of tumor cells in a given core was calculated using the Ariol platform (Genetix Corp.). ERG expression was calculated on 5 μm tissue microarray sections (91% of patients) and prostate tissue block sections (9% of patients), using rabbit monoclonal anti-ERG antibody (Clone ID: EPR3864; Epitomics, Inc.) at a dilution of 1:100. Tumor specimens were evaluated individually by a study pathologist (R. Lis). The presence of ERG staining in the vascular endothelium served as a positive internal control, with ERG assessment restricted to cores in which the positive internal control was observed. A patient was considered positive for tumor ERG expression if ERG staining was observed within prostate cancer epithelial cells of at least one tissue microarray core.

Biomarkers of angiogenesis. Angiogenesis markers were assessed on 5 μm serial sections of the individual prostate tissue blocks in the HPFS cohort only. One to nine blocks with cancer were evaluated per case by a study pathologist as previously described by Mucci and colleagues (39). Endothelial cell marker CD34 protein expression was visualized using immunohistochemistry (QBEND10 primary mouse monoclonal antibody; Biogenex) and imaged using Image ProPlus 4.5 software (Media Cybernetics), a semiautomated image analysis platform. Angiogenesis markers were defined as the following: microvessel density, that is, the number of vascular structures in a high-power field (×200); vessel area in μm^2; vessel diameter in μm; and vessel irregularity, that is, the irregularity of the vessel lumen calculated as the $\text{perimeter}^2/4 \cdot \Pi \times$ area, where a value of 1.0 indicates a perfect circle and values >1.0 indicate increasing irregularity. Measurements were averaged over the total tumor area evaluated for each patient. Smaller vessel area and diameter, and less regular vessel shape were associated with development of lethal prostate cancer in this cohort (39).

Statistical analysis

Analyses were based on the 902 men ($n = 346$ from PHS; $n = 556$ from HPFS) for whom PSMA expression was measured. The average value of each biomarker was calculated across all cores or tumor sections for a given patient. We compared age at diagnosis, clinical parameters, and BMI across quartiles of PSMA expression using ANOVA for normally distributed continuous variables, Kruskal–Wallis test for non-normally distributed continuous measures, and χ^2 tests for categorical variables.

Cox proportional hazards regression was used to calculate multivariable HRs and 95% confidence intervals (CI) for the association between PSMA expression and lethal prostate cancer. Follow-up time was calculated from the date of diagnosis to development of distant metastases, death from prostate cancer, or censored at death from another cause or end of follow-up (January 2009 or last date of contact for PHS; April 2012 for HPFS), whichever occurred first. We adjusted for tissue microarray (indicator variables) to account for staining variation across microarrays, and age at diagnosis (continuous), in all models. We further adjusted for Gleason score (2 to 6, 3 + 4, 4 + 3, 8 to 10) and PSA at diagnosis (<4, 4 to <10, ≥10 ng/mL, missing) to test whether PSMA expression was an independent predictor of lethal prostate cancer risk. We also examined these associations stratified by tumor stage (T1–T2, N0–Nx, M0–Mx vs. T3–T4 or N1 or M1), Gleason score (2 to 7 vs. 8 to 10), and ERG expression (absent, present). Violation of the proportional hazards assumption was tested by creating interaction terms between PSMA quartiles and follow-up time; the addition of the interaction terms to the model including PSMA quartiles, age at diagnosis, and tissue microarray, was not statistically significant (Wald test $P = 0.21$; 3 degrees of

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freedom), thus the assumption was satisfied. Because PSMA is negatively correlated with androgen levels (1, 22), we also performed a sensitivity analysis excluding the 57 patients who received any type of neoadjuvant or adjuvant hormone therapy ± 1 year from the date of radical prostatectomy or TURP. To test the association between PSMA expression and the composite endpoint of biochemical recurrence and lethal disease, follow-up time was calculated from date of diagnosis to date of recurrence, distant metastases, or death from prostate cancer; patients without a recurrence were censored at death from another cause or end of follow-up.

We examined correlations between PSMA expression and tumor biomarkers (proliferation index, apoptotic index, AR expression, VDR expression, and angiogenesis measures) using partial Spearman rank correlations, adjusted for age at diagnosis, and tissue microarray. PSMA expression across categories of ERG expression (absent, present) was evaluated using analysis of covariance (ANCOVA), adjusted for age at diagnosis and tissue microarray.

Analyses were conducted using SAS system software (version 9.2; SAS Institute). All P values were two-sided and considered statistically significant if less than 0.05.

Results

Among the 902 patients with prostate cancer, mean age at diagnosis was 65.8 years with an average follow-up time of 13.2 years (Table 1). Higher PSMA expression was associated ($P < 0.01$) with increasing age, higher Gleason score, and higher PSA at diagnosis, and modestly associated ($P = 0.07$) with higher tumor stage. Mean tumor PSMA expression among all patients was 43.9 with an interquartile range (IQR) of 10.5 to 70.7. PSMA expression (mean ± SD) was similar between the cohorts (44.7 ± 36.8 for PHS and 43.5 ± 35.7 for HPFS), and between prostatectomy and TURP specimens (44.2 ± 36.1 and 39.6 ± 36.4, respectively). PSMA staining in the tumor was membranous and cytoplasmic (Fig. 1).

PSMA protein expression in tumor tissue was associated with a 2.4-fold (95% CI, 1.3–4.5) increased risk of lethal prostate cancer comparing the highest to lowest quartile, adjusting for age at diagnosis, and tissue microarray (Table 2). This positive association was stronger among patients with nonadvanced stage disease ($HR_{Quartile(Q)4\ vs.\ 1}$, 4.3; $P_{trend} < 0.01$), lower Gleason score ≤7 tumors ($HR_{Q4\ vs.\ 1} = 4.6$; $P_{trend} < 0.01$), as well as those with ERG-positive tumors ($HR_{Q4\ vs.\ 1} = 3.5$; $P_{trend} < 0.01$). No associations with lethal cancer were found in men with advanced stage disease ($P_{trend} = 0.27$), poorly differentiated (Gleason score ≥8) tumors ($P_{trend} = 0.39$), or ERG-negative tumors ($P_{trend} = 0.35$). After further adjustment for Gleason score and PSA at diagnosis, the associations between PSMA expression and lethal prostate cancer were attenuated for overall ($P_{trend} = 0.76$), nonadvanced ($P_{trend} = 0.61$), Gleason score ≤7 ($P_{trend} = 0.51$), and ERG-positive ($P_{trend} = 0.88$) prostate cancer, and all were nonsignificant.

Among all 902 patients, associations of clinical parameters and risk of lethal prostate cancer were: age at diagnosis (per 5-year increase; HR, 1.2; 95% CI, 1.0–1.4); Gleason score ($HR_{3+4\ vs.\ 2–6}$, 1.4; 95% CI, 0.5–4.5; $HR_{4+3\ vs.\ 2–6}$, 4.1; 95% CI, 1.4–12.0; $HR_{8–10\ vs.\ 2–6}$, 7.7; 95% CI, 2.7–21.9); PSA at diagnosis ($HR_{4–9.9\ vs.\ <4}$, 1.5; 95% CI, 0.3–6.2; $HR_{\geq 10\ vs.\ <4}$, 2.8; 95% CI, 0.7–11.8); tumor stage ($HR_{T3\ vs.\ T1–T2}$, 1.7; 95% CI, 1.1–2.8; $HR_{T4/N1/M1\ vs.\ T1–T2}$, 5.1; 95% CI, 2.9–9.1); mutually adjusted for all four parameters.

In the model adjusting for age at diagnosis and tissue microarray, effect estimates were slightly stronger after excluding patients who had received neoadjuvant or adjuvant hormone therapy: $HR_{Q2\ vs.\ 1}$, 2.14 (95% CI, 1.03–4.44), $HR_{Q3\ vs.\ 1}$, 2.01 (95% CI, 0.96–4.21), $HR_{Q4\ vs.\ 1}$, 3.20 (95% CI, 1.60–6.39), $P_{trend} < 0.01$. Similar to the main analysis, results were attenuated and nonsignificant after further adjusting for Gleason score and PSA at diagnosis: $HR_{Q2\ vs.\ 1}$, 1.78 (95% CI, 0.84–3.80), $HR_{Q3\ vs.\ 1}$, 1.72 (95% CI, 0.80–3.72), $HR_{Q4\ vs.\ 1}$, 1.38 (95% CI, 0.67–2.86), $P_{trend} = 0.92$.

Compared with the primary outcome of lethal prostate cancer, the association between PSMA expression and the composite outcome of biochemical recurrence and lethal disease was weaker and nonsignificant: $HR_{Q2\ vs.\ 1}$, 0.90 (95% CI, 0.61–1.33), $HR_{Q3\ vs.\ 1}$, 1.26 (95% CI, 0.87–1.82), $HR_{Q4\ vs.\ 1}$, 1.24 (95% CI, 0.86–1.78), $P_{trend} = 0.09$, adjusting for age at diagnosis and tissue microarray; and $HR_{Q2\ vs.\ 1}$, 0.75 (95% CI, 0.50–1.12), $HR_{Q3\ vs.\ 1}$, 0.89 (95% CI, 0.61–1.31), $HR_{Q4\ vs.\ 1}$, 0.68 (95% CI, 0.46–1.01), $P_{trend} = 0.13$, after further adjusting for Gleason score and PSA at diagnosis.

Tumors with high PSMA expression showed significantly lower protein expression of VDR and AR, and absence of ERG protein expression, among all patients (Table 3). High PSMA expression was also significantly correlated with markers of angiogenic activity, including higher microvessel density, smaller vessel area, smaller vessel diameter, and irregular shape. With the exception of ERG expression, the correlations between PSMA and other tumor biomarkers did not retain statistical significance in poorly differentiated tumors. No correlations were found for proliferation or apoptotic indices among all patients or within subgroups.

The association between PSMA expression and lethal prostate cancer among all patients, adjusted for age at diagnosis and tissue microarray, remained statistically significant after further adjustment for VDR ($HR_{Q4\ vs.\ 1}$, 2.16; 95% CI, 1.14–4.11; $P_{trend} = 0.03$; $n = 812$), AR ($HR_{Q4\ vs.\ 1}$, 2.31; 95% CI, 1.25–4.29; $P_{trend} < 0.01$; $n = 860$), or ERG expression ($HR_{Q4\ vs.\ 1}$, 2.41; 95% CI, 1.28–4.53; $P_{trend} < 0.01$; $n = 880$). Among HPFS patients with measured angiogenesis markers (microvessel density, vessel area, vessel diameter, and vessel irregularity), higher PSMA expression was nonsignificantly associated with lethal disease ($HR_{Q4\ vs.\ 1}$, 2.45; 95% CI, 0.92–6.49; $P_{trend} = 0.19$; $n = 414$), adjusting for age at diagnosis and tissue microarray. This association was attenuated after further adjusting for all four markers ($HR_{Q4\ vs.\ 1}$, 1.65; 95%

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Table 1. Characteristics of 902 men with prostate cancer in the PHS and HPFS according to PSMA expression in tumor tissue

		PSMA quartile (Q)				
	All patients	**Q1 (low)**	**Q2**	**Q3**	**Q4 (high)**	***P***
N cases	902	225	226	226	225	
Mean (SD) age at diagnosis, y	65.8 (6.3)	65.1 (6.4)	66.2 (6.3)	65.2 (6.7)	66.8 (5.6)	<0.01[a]
Mean (SD) follow-up time, y	13.2 (5.0)	13.6 (5.1)	13.1 (4.9)	13.4 (5.0)	12.6 (4.8)	0.13[a]
Tumor stage, *N* (%)						
T1–T2, N0–Nx, M0–Mx	640 (71.0)	173 (76.9)	166 (73.5)	144 (63.7)	157 (69.8)	0.07[b]
T3, N0–Nx, M0–Mx	222 (24.6)	45 (20.0)	49 (21.7)	70 (31.0)	58 (25.8)	
T4 or N1 or M1	38 (4.2)	6 (2.7)	11 (4.9)	12 (5.3)	9 (4.0)	
Missing	2 (0.2)	1 (0.4)	0	0	1 (0.4)	
Gleason score, *N* (%)						
2–6	178 (19.7)	70 (31.1)	57 (25.2)	36 (15.9)	15 (6.7)	<0.01[c]
3 + 4	335 (37.1)	100 (44.4)	84 (37.2)	82 (36.3)	69 (30.7)	
4 + 3	223 (24.7)	29 (12.9)	52 (23.0)	64 (28.3)	73 (34.7)	
8–10	166 (18.4)	26 (11.6)	33 (14.6)	44 (19.5)	63 (28.0)	
PSA at diagnosis, ng/mL						
Median (IQR)	7.0 (5.0,11.0)	7.0 (4.8,9.9)	6.5 (4.7,9.3)	7.5 (5.0,12.5)	7.6 (5.5,13.0)	<0.01[d]
Categories, *N* (%)						
<4	87 (9.7)	26 (11.6)	28 (12.4)	21 (9.3)	12 (5.3)	0.01[b]
4 to <10	449 (49.8)	118 (52.4)	120 (53.1)	109 (48.2)	102 (45.3)	
≥10	231 (25.6)	47 (20.9)	48 (21.2)	65 (28.8)	71 (31.6)	
Missing	135 (15.0)	34 (15.1)	30 (13.3)	31 (13.7)	40 (17.8)	
BMI at diagnosis, kg/m^2						
Mean (SD)	25.6 (3.4)	25.6 (3.3)	25.7 (4.2)	25.5 (3.0)	25.6 (3.1)	0.91[a]
Categories, *N* (%)						
< 25	379 (42.0)	96 (42.7)	104 (46.0)	86 (38.1)	93 (41.3)	0.40[b]
25 to <28	276 (30.6)	73 (32.4)	64 (28.3)	81 (35.8)	58 (25.8)	
≥28	155 (17.2)	39 (17.3)	39 (17.3)	35 (15.5)	42 (18.7)	
Missing	92 (10.2)	17 (7.6)	19 (8.4)	24 (10.6)	32 (14.2)	

[a]ANOVA test; 3 degrees of freedom. Excluded individuals with missing values.
[b]Chi-square test; 6 degrees of freedom. Excluded individuals with missing values.
[c]Chi-square test; 9 degrees of freedom.
[d]Kruskal–Wallis test; 3 degrees of freedom. Excluded individuals with missing values.

CI, 0.60–4.54; $P_{trend} = 0.75$), or any of the markers individually (data not shown).

Discussion

In a large cohort of prostate cancer patients with over 13 years of average follow-up, PSMA protein expression in tumor tissue was positively associated with risk of lethal disease, but this association was not independent of clinical parameters. Thus, our study does not support the clinical utility of PSMA expression as a strong candidate biomarker for lethal prostate cancer among surgically treated patients. After considering additional markers of disease aggressiveness, we found that PSMA expression likely captures, in part, malignant features of Gleason grade and tumor angiogenesis.

Three prior studies of PSMA protein expression in prostate tumor tissue have reported positive associations with risk of biochemical recurrence (5–7). Minner and colleagues followed 1,426 patients with prostate cancer for up to 12 years and noted a borderline significant association for high versus low PSMA expression in radical prostatectomy tissue and PSA recurrence (7). Similar to our study, the association did not remain statistically significant after multivariable adjustment for clinical parameters. A smaller study of 136 patients (61% with organ-confined tumors) who underwent radical prostatectomy found that PSMA overexpression was associated with biochemical recurrence, even after multivariable adjustment for clinicopathological parameters (6). A third study of 93 patients (43% with lymph-node positive disease at surgery) found a significant positive association between PSMA expression and biochemical recurrence after adjusting for extraprostatic extension, though the estimates adjusted for additional clinical parameters is not presented (5). Our results may differ from these studies as

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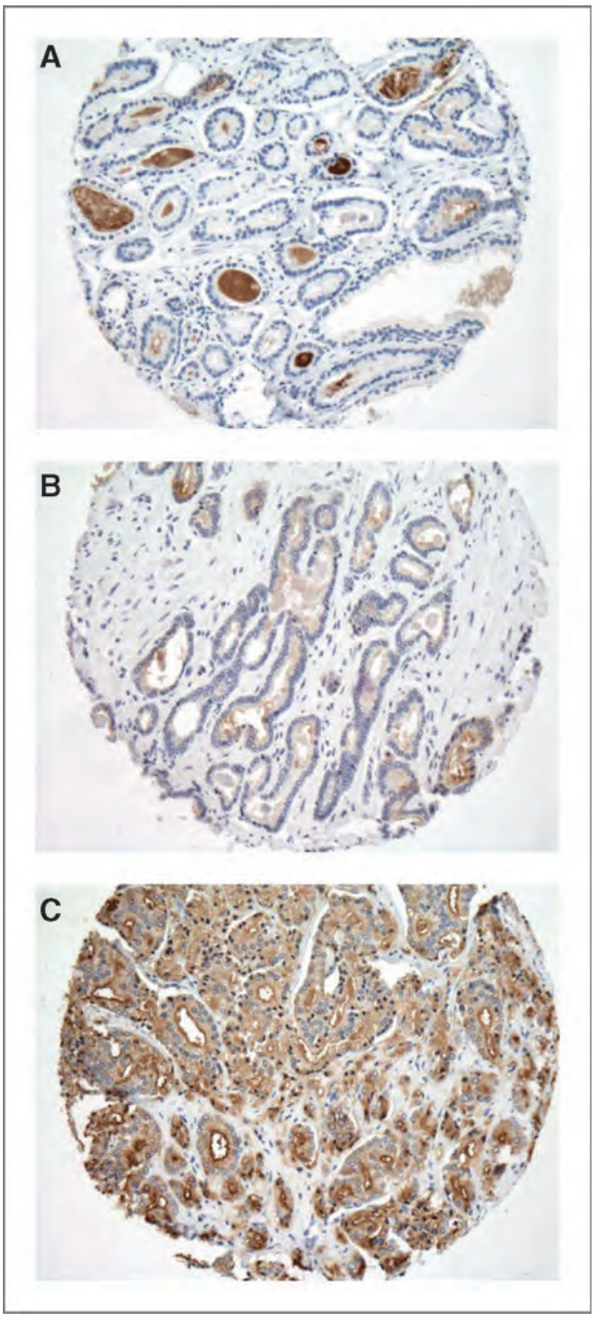

Figure 1. Representative images of PSMA protein expression in selected prostate tumor tissue microarray cores from the HPFS: (A) weak staining in a patient with Gleason score 3 + 3 tumor; (B) moderate staining in a patient with Gleason score 3 + 4 tumor; and (C) strong staining in a patient with Gleason score 4 + 4 tumor. Images were taken at ×20 magnification. The prostate tumor glands showed cytoplasmic and membranous staining.

more than 70% of our patients were diagnosed with nonadvanced stage tumors, our specimens were re-reviewed by a study pathologist for uniformity of Gleason score, and PSA levels at diagnosis were included in the multivariable models. Because PSMA expression has been positively correlated with these clinicopathologic features, it is unclear whether the positive findings from other studies would persist after accounting for all these factors. Furthermore, Ross and colleagues used the 7E11 anti-PSMA antibody, which recognizes the internal domain of PSMA (6), whereas the other two prior studies (5, 7) and our current study used clone 3E6, which recognizes the extracellular domain. Finally, our results may differ as our study was the first to assess lethal disease as the primary endpoint, whereas all prior studies evaluated time to biochemical recurrence.

We previously showed that a greater number of smaller and more poorly formed vessels within the prostate tumor were strong predictors of lethal disease (39). Our current study supports that PSMA is indicative of increased tumor angiogenesis, and after adjusting for these markers, the association of PSMA expression with lethal prostate cancer was markedly attenuated. This is consistent with the prior observation of PSMA being expressed in the endothelial cells of certain solid tumor neovasculature, including prostate cancer, renal cell carcinoma, transitional cell carcinoma of the bladder, gastric cancer, and colorectal cancer (27–30). Also, a small study of LNCaP tumors grown in nude mice found a strong positive correlation between protein expression of PSMA and VEGF, a signal protein that stimulates angiogenesis (40).

PSMA seems to be regulated by androgens, in that PSMA expression in prostate tumors is highest in hormone-deprived states, and is repressed in response to testosterone (1, 22). We found that higher PSMA expression was correlated with lower AR expression in prostate tumor tissue, though we did not have a measure of circulating testosterone levels at the time of surgery in our study. We also found that PSMA expression was lower in tumors that expressed ERG, which is supported by the prior finding that *TMPRSS2-ERG* fusion negatively regulated PSMA expression in LNCaP cells (24). In addition, the association between PSMA expression and lethal prostate cancer in our study was limited to ERG-positive tumors, suggesting that the link between PSMA and disease progression may depend on the molecular subtype of the tumor. Further studies are warranted to better understand the mechanisms by which PSMA, AR, and the *TMPRSS2-ERG* fusion may interact to influence prostate carcinogenesis.

The negative correlation we observed between VDR and PSMA expression is consistent with Serda and colleagues, who reported that 1α,25-dihydroxyvitamin D_3 downregulated PSMA expression in LNCaP cells (19). We previously reported an inverse association between VDR expression and prostate cancer progression in this patient cohort (37). In the current study, PSMA expression was associated with lethal prostate cancer independently of VDR levels in the age- and tissue microarray–adjusted models, suggesting that PSMA and VDR may act through different mechanisms to influence disease progression. Indeed, vitamin D has been shown to exert antiproliferative and proapoptotic effects on prostate tumors (20,

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Table 2. HRs and 95% CIs for the association between PSMA expression in tumor tissue and lethal prostate cancer

	PSMA quartile (Q)				
	Q1 (low)	Q2	Q3	Q4 (high)	P_{trend}[a]
All patients					
N lethal events	15	24	22	34	
N censored	210	202	204	191	
Person-time, y	3,061	2,971	3,032	2,825	
Model 1[b]	1.00	1.64 (0.85,3.14)	1.55 (0.80,3.01)	2.42 (1.31,4.48)	<0.01
Model 2[c]	1.00	1.17 (0.60,2.30)	1.11 (0.56,2.22)	1.01 (0.52,1.93)	0.76
Nonadvanced stage[d]					
N lethal events	4	10	8	16	
N censored	169	156	136	141	
Person-time, y	2,393	2,277	1,958	2,007	
Model 1[b]	1.00	2.43 (0.75,7.83)	2.42 (0.73,8.07)	4.34 (1.43,13.12)	<0.01
Model 2[c]	1.00	1.86 (0.55,6.30)	2.06 (0.60,7.06)	1.74 (0.53,5.73)	0.61
Advanced stage[e]					
N lethal events	10	14	14	18	
N censored	41	46	68	49	
Person-time, y	661	693	1,074	810	
Model 1[b]	1.00	1.35 (0.59,3.09)	1.17 (0.50,2.74)	1.65 (0.74,3.64)	0.27
Model 2[c]	1.00	0.90 (0.38,2.11)	0.85 (0.34,2.09)	0.78 (0.34,1.78)	0.55
Gleason score 2–7					
N lethal events	5	13	11	16	
N censored	194	180	171	146	
Person-time, y	2,808	2,591	2,504	2,109	
Model 1[b]	1.00	3.05 (1.08,8.65)	2.62 (0.91,7.59)	4.63 (1.68,12.73)	<0.01
Model 2[c]	1.00	2.64 (0.90,7.73)	2.02 (0.67,6.11)	2.11 (0.72,6.17)	0.51
Gleason score 8–10					
N lethal events	10	11	11	18	
N censored	16	22	33	45	
Person-time, y	253	380	528	716	
Model 1[b]	1.00	0.51 (0.21,1.27)	0.60 (0.24,1.47)	0.53 (0.23,1.25)	0.39
Model 2[c]	1.00	0.56 (0.22,1.45)	0.78 (0.30,2.03)	0.59 (0.24,1.40)	0.47

[a]Wald test modeling the median expression values for each PSMA quartile.
[b]Adjusted for age at diagnosis (continuous) and tissue microarray.
[c]In addition adjusted for Gleason score (2 to 6, 3 + 4, 4 + 3, 8–10), and PSA at diagnosis (<4, 4 to <10, ≥10 ng/mL, missing).
[d]Tumor stage T1–T2, N0–Nx, M0–Mx.
[e]Tumor stage T3–T4, or N1 or M1.

21, 41), whereas we found no correlation between PSMA and indices of proliferation or apoptosis.

Limitations of our study include potential misclassification of PSMA protein expression due to assay and detection variability, though any bias is likely nondifferential as study pathologists were blinded to outcome status. Also, we had low statistical power to detect associations among subgroups of patients with small numbers of outcomes. Furthermore, we used mainly prostatectomy tissue with the majority of patients having organ-confined disease, thus it is unknown whether our findings would be generalizable to PSMA expression measured in biopsy specimens. Our study has several notable strengths. We were the first to evaluate the association between PSMA expression and lethal disease within two large, established cohort studies with long-term and complete follow-up among patients with prostate cancer. In addition, the patients were well-characterized with respect to clinical and pathologic measures, including re-review of Gleason scores.

In our study of 902 U.S.-based patients with prostate cancer, PSMA protein expression measured in prostate tumor tissue was associated with progression to lethal disease, but not independent of clinical predictors. Our results suggest that PSMA is an indicator of increased tumor angiogenesis, and through this pathway, increased risk of prostate cancer progression. Overall, our findings do not support the clinical utility of tumor PSMA expression as a predictor of lethal disease among patients who

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Table 3. Correlation of PSMA protein expression in prostate tumor tissue with other tumor biomarkers

	All patients	Nonadvanced stage[a]	Advanced stage[b]	Gleason score 2–7	Gleason score 8–10
Partial Spearman rank correlation coefficients[c]					
Proliferation index					
N	867	613	252	707	160
Median [Q1, Q3]	0.13 [0, 0.55]	0.14 [0, 0.56]	0.12 [0, 0.49]	0.11 [0, 0.46]	0.23 [0.03, 1.01]
r	−0.00002	−0.001	0.009	0.004	−0.127
P	1.00	0.98	0.89	0.93	0.12
Apoptosis index					
N	716	507	208	589	127
Median [Q1, Q3]	0.50 [0, 2.00]	0.50 [0, 2.00]	0.50 [0, 2.00]	0.50 [0, 2.00]	0.50 [0, 2.00]
r	−0.005	−0.004	0.015	0.038	−0.166
P	0.89	0.93	0.83	0.37	0.07
VDR protein expression					
N	812	567	243	658	154
Median [Q1, Q3]	29.1 [13.0, 45.4]	31.6 [14.9, 47.7]	24.0 [8.9, 42.8]	30.9 [14.3, 47.7]	21.0 [7.0, 37.7]
r	−0.084	−0.098	−0.010	−0.066	−0.049
P	0.02	0.02	0.87	0.09	0.56
AR protein expression					
N	860	612	246	704	156
Median [Q1, Q3]	117.7 [112.3, 123.0]	117.3 [112.3, 123.0]	117.7 [111.0, 123.0]	115.0 [111.0, 123.0]	117.7 [112.3, 123.0]
r	−0.103	−0.099	−0.123	−0.100	−0.144
P	<0.01	0.01	0.06	<0.01	0.08
Markers of angiogenesis[d]					
Microvessel density					
N	414	275	139	332	82
Median [Q1, Q3]	67.1 [55.0, 95.0]	65.3 [53.0, 92.5]	74.3 [58.0, 100.0]	66.6 [52.9, 93.0]	75.5 [59.0, 102.7]
r	0.162	0.165	0.168	0.167	0.011
P	<0.01	<0.01	0.05	<0.01	0.93
Vessel area					
N	415	276	139	332	83
Median [Q1, Q3]	466.5 [357.7, 654.7]	486.5 [370.5, 664.4]	430.2 [304.6, 648.7]	485.0 [371.9, 671.6]	420.0 [301.3, 567.4]
r	−0.168	−0.165	−0.198	−0.147	−0.150
P	<0.01	<0.01	0.02	<0.01	0.19
Vessel diameter					
N	415	276	139	332	83
Median [Q1, Q3]	24.2 [21.4, 27.8]	24.4 [21.9, 27.7]	23.3 [20.3, 27.9]	24.5 [21.8, 28.3]	22.6 [19.8, 26.2]
r	−0.141	−0.130	−0.192	−0.120	−0.119
P	<0.01	0.03	0.03	0.03	0.30
Vessel irregularity[e]					
N	415	276	139	332	83
Median [Q1, Q3]	4.0 [3.2, 4.8]	3.9 [3.1, 4.7]	4.1 [3.3, 4.9]	3.9 [3.2, 4.7]	4.1 [3.4, 5.1]
r	0.100	0.026	0.250	0.057	0.124
P	0.04	0.68	<0.01	0.31	0.28
ANCOVA[c]					
ERG expression					
Absent, *N*	434	322	111	348	86
Adjusted mean PSMA	64.2	67.5	64.8	59.0	72.6
Present, *N*	446	301	144	366	80
Adjusted mean PSMA	49.3	51.7	50.3	44.0	59.1
P	<0.01	<0.01	<0.01	<0.01	0.02

[a]Tumor stage T1–T2, N0–Nx, M0–Mx.
[b]Tumor stage T3–T4 or N1 or M1.
[c]Adjusted for age at diagnosis and tissue microarray.
[d]Measured in HPFS cohort only.
[e]Higher score indicates more irregularity.

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undergo radical prostatectomy, though it is unknown how this biomarker may perform in biopsy specimens from patients who choose other treatment modalities such as active surveillance or radiation.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): J.L. Kasperzyk, S.P. Finn, R. Flavin, M. Fiorentino, R. Lis, W.K. Hendrickson, S.K. Clinton, H.D. Sesso, E.L. Giovannucci, M. Loda, L.A. Mucci
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Original Study

GermLine Variation in Superoxide Dismutase-2 (*SOD2*) and Survival Outcomes After Radiation Therapy for Prostate Cancer: Results of a Test and Validation Set Analysis

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Abstract

In this study, we investigated whether patient-specific differences in the antioxidant gene, superoxide dismutase-2 (*SOD2*), affect the efficacy of radiation therapy for prostate cancer. We identified a link between common mutations in the *SOD2* gene and prostate cancer recurrence after radiation for prostate cancer in a group of predominantly low-risk prostate cancer patients but not in a higher-risk cohort.

Background: Genetic variants in antioxidant pathways might decrease the efficacy of radiation therapy (RT) by suppressing the generation of reactive oxygen species. We studied the association between single nucleotide polymorphisms (SNPs) in the antioxidant gene superoxide dismutase-2 (*SOD2*) and cancer-specific outcomes after RT. **Patients and Methods:** Among 816 prostate cancer patients who received radiation as primary therapy from the Physicians' Health Study and the Health Professionals Follow-up Study, we evaluated the association of 7 tagging SNPs in *SOD2* with lethal prostate cancer (death from prostate cancer or distant metastasis among living patients). We sought to validate findings in a separate cohort of 612 prostate cancer patients treated with RT with a greater proportion of intermediate and high-risk Gleason scores at the Dana-Farber Cancer Institute. Genetic effects were analyzed using a codominant model, using the genotype homozygous for the major allele as baseline. **Results:** Among patients who underwent RT in the test cohort, there was a significant association between 3 of the 7 *SOD2* SNPs and lethal prostate cancer: rs6917589 (overall $P = .006$), rs2758331 ($P = .04$) and the functional valine to alanine polymorphism in rs4880 ($P = .04$). These SNPs were not associated with outcome among men who had undergone prostatectomy. The associations were not replicated in the validation cohort. **Conclusion:** Germline genetic variation in the *SOD2* gene might be a predictive biomarker of response to RT for prostate cancer but is not consistently associated with outcome after RT across prostate cancer cohorts with different clinical characteristics.

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Introduction

Germline variation in antioxidant pathways might alter the effect of cancer therapies that rely on the generation of cytotoxic reactive oxygen species (ROS). Somatic alterations in the antioxidant environment are also postulated to result in enhanced cancer cell survival.[1] There is growing interest in molecular-based strategies that target antioxidant pathways to promote cancer cell killing via oxidative stress.[2-4] Radiation therapy (RT) generates ROS that mediate DNA damage and other downstream effects on cancer cells.[5] Patient germline variability in endogenous antioxidant enzymes involved in neutralizing ROS might explain variability in cancer-specific outcomes after RT. For example, patients with increased capacity for neutralizing ROS might receive less benefit from RT compared with patients with an impaired ability to neutralize cytotoxic ROS.

Superoxide dismutase (SOD)-2 is a mitochondrial antioxidant enzyme that is an important ROS scavenger. SOD2 reduces superoxide anion to hydrogen peroxide and oxygen, which is then converted to water by catalase (CAT) and glutathione peroxidase (GPX; Figure 1). Overexpression of mitochondrial *SOD* was previously shown to protect cells from radiation-induced neoplastic transformation[6] and decreased levels of SOD increased the radiosensitivity of prostate cancer cells in vitro.[7] A specific polymorphism in codon 16 of *SOD2*, rs4880, results in a valine to alanine amino acid change and is postulated to decrease mitochondrial ROS by causing more efficient transport of the enzyme into the mitochondria.[8,9] The polymorphism would be expected to decrease the effectiveness of cancer therapies such as RT, which rely on formation of ROS. Polymorphisms in *SOD2* were previously shown to be associated with late toxicity after RT for prostate cancer,[10] breast cancer,[11] and head and neck cancer.[12]

There are conflicting data on the prognostic significance of *SOD2* polymorphisms and survival after cancer therapy.[13,14] In this study we sought to validate the association between *SOD2* polymorphisms and cancer outcomes after RT for prostate cancer. We hypothesized that germline genetic variation in *SOD2* is associated with outcome after RT and that the functional rs4880 polymorphism is associated with adverse prostate cancer outcomes.

Patients and Methods

Patients and Outcomes

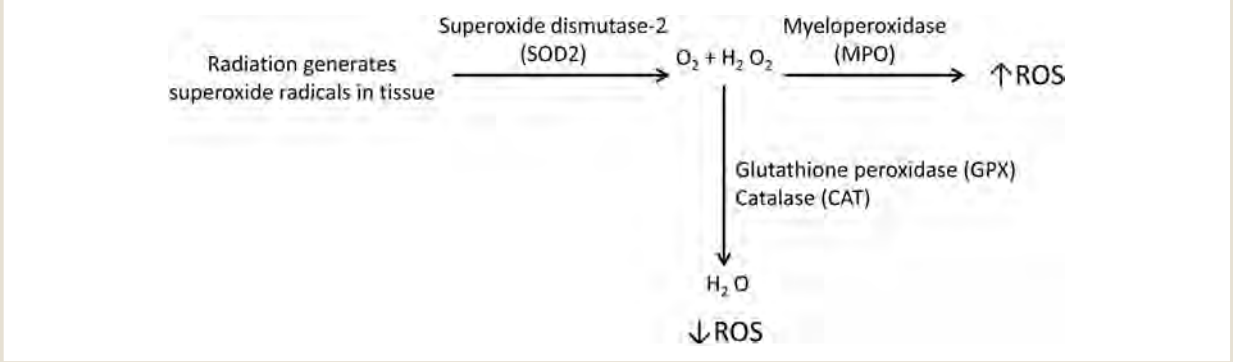
The test cohort was comprised of 816 participants from 2 prospective cohort studies, the Physicians' Health Study (PHS; 1982-2009, n = 387) and the Health Professionals Follow-up Study (HPFS; 1993-2010, n = 429). The PHS[15-17] was a 2 × 2 randomized double-blind, placebo-controlled trial that began in 1982 and enrolled 22,071 US male physicians ages 40-84 years to take 325 mg aspirin and/or 50 mg beta-carotene every other day or placebo. Participants were free from diagnosed cancer at enrollment and were followed with yearly questionnaires and postcards at 6-month intervals to ascertain end points, including prostate cancer. At baseline, 14,916 (68%) participants provided blood before randomization and cancer diagnosis. The prospective HPFS enrolled 51,529 male medical professionals in 1986 to investigate the causes of cancer and heart disease. These cohort participants are subsequently followed with biennial questionnaires designed to collect information about medical diagnoses and lifestyle factors. Response rates to the follow-up surveys are high at approximately 96% and 18,018 participants provided a blood sample between 1993 and 1995.

When a participant reported a diagnosis of prostate cancer, hospital records and pathology reports were requested and study physicians verified diagnosis by reviewing medical records and pathology reports to determine the Gleason grade, stage, and prostate-specific antigen (PSA) level at diagnosis. The present study included men in the PHS and HPFS blood cohorts who were diagnosed with prostate cancer between 1982 and 2010 and who underwent RT. Participants were excluded if RT was not their primary treatment or if their first treatment was radical prostatectomy.

For comparison, we also analyzed the association of polymorphisms in *SOD2* in patients who underwent radical prostatectomy without RT, reasoning that the genetic variations would have no effect after surgical intervention. This separate cohort included 1094 patients from the PHS (n = 555) and the HPFS (n = 539). These studies were approved by the institutional review board at the Harvard School of Public Health and Partners Health Care.

The validation cohort consisted of patients from the Prostate Clinical Research Information System (CRIS; 1990-2008,

Figure 1 Simplified Schema of the Relationship Between SOD2, Reactive Oxygen Species (ROS), and Other Enzymes Involved in Free-Radical Scavenging in Tissue



n = 612) at the Dana-Farber Cancer Institute (DFCI). CRIS consists of a central secure data repository of patient data, including baseline clinical and disease characteristics and information about treatment and outcomes. All prostate cancer patients at Dana Farber Cancer Institute and Brigham and Women's Hospital were offered enrollment and 647 patients were initially identified for the validation cohort. Selected patients had prostate cancer, were treated with external beam radiation or brachytherapy, consented to provide information and tissue, and donated blood for research purposes. Patients were excluded if they had lymph node or distant metastases before RT, or if the samples failed > 50% of the genotyping assays.

For the test cohort, the primary outcome was time to development of lethal prostate cancer, defined as the time from initiation of RT to prostate cancer-specific death or distant metastasis among living participants. Outcomes, including cause of death, were verified via death certificates and medical record review. Because it was not routinely verified in the PHS, we did not use biochemical recurrence as an outcome in the test cohort. For the validation cohort, in the primary analysis we evaluated the association between single nucleotide polymorphism (SNP) genotypes and time to distant metastasis, which was defined as the time from the initiation of RT to the time when metastases developed. Because of shorter follow-up in the validation cohort, prostate cancer death was not used as the primary outcome. As a secondary analysis, we also evaluated the association with time to biochemical recurrence. Time to biochemical recurrence was defined as the time from the start of RT to the time when nadir + 2 ng/mL occurred or to time of salvage therapy. If the outcome of interest did not occur, follow-up was censored on the last PSA date.

Genotyping

We characterized 1 candidate SNP (rs4880) and 6 tagging SNPs from *SOD2* that were selected to capture genetic variation across the *SOD2* gene, including 5 kb upstream and downstream, with an average $r^2 > 0.80$ (Tagger; *http://www.broadinstitute.org/mpg/tagger/*, using HapMap Release 21, CEU analysis panel: Utah residents of Northern and Western European Ancestry). For the test cohort, genotyping was performed at the Harvard Medical School Partners Healthcare Center for Genetics and Genomics after extraction of DNA from whole blood using Biotrove Open Genetics and Genomics with a standard QIAmp kit (Qiagen, Chatsworth, CA) protocol. All SNPs had > 90% completion and the concordance was > 99% for blinded quality control samples. All SNPs were in Hardy–Weinberg equilibrium.

For the validation cohort, all DNA samples were extracted from patients' peripheral whole blood using the QIAamp DNA Blood mini kit (Qiagen) according to the manufacturer's instruction. Genotyping was performed at the core facility of Boston Children's Hospital using Sequenom iPLEX matrix-assisted laser desorption/ionization time-of-flight mass spectrometry technology. Approximately 5% of randomly selected duplicates were included as the quality control. All SNPs had > 99% genotype passing rates and no discrepancy between duplicates was observed in the genotyping data. Laboratory personnel were blinded to all case status information.

Statistical Methods

Patient clinical and disease characteristics at the time of diagnosis were summarized according to median and IQR for continuous variables and number and percentage for categorical variables. For the test and validation cohorts, we analyzed the genetic effects of *SOD2* SNPs using the codominant model, in which the heterozygous and homozygous minor allele genotypes were treated as separate categories and compared with the homozygous major allele genotype. For minor alleles with < 10% frequency in the cohorts, we combined the minor homozygous with the heterozygous genotypes. The codominant model was used because it makes fewer assumptions about the nature of the effect of the minor allele on outcome compared with the additive model.

Cox proportional hazards models were used to assess the unadjusted and adjusted association between SNP and outcome and were used to calculate hazard ratios (HRs) and associated 95% confidence intervals (CIs). The adjusted models included biopsy Gleason score, log-transformed PSA at diagnosis, clinical stage, and age at treatment. The median age at the time of diagnosis and treatment were the same. For the test cohort, year of diagnosis and cohort (PHS or HPFS) were also used as adjustment covariates, and missing values for the clinical variables used in the adjusted models were imputed using Multiple Imputation for Chained Equations in *R*. The use of hormonal therapy was included in the adjusted model for only the validation cohort.

All reported P values are 2-sided, with Bonferroni-corrected $P < .007$ considered statistically significant and $P < .05$ considered nominally significant. SAS version 9.3 (SAS Institute, Cary, NC) and *R* version 3.0.2 were used for all analyses.

Results

In Table 1 the patient characteristics from the test (n = 816) and validation cohorts (n = 612) are shown. Patients in the test cohort were older (median age 73 vs. 64 years in the validation cohort) and had longer follow-up compared with the validation cohort (median 10.2 years vs. 6.8 years). They were more likely to have low grade Gleason score ≤ 6 tumors (60%) and to be treated in an earlier time period than the validation cohort in which most patients had higher-risk Gleason scores (≥ 7; 43%) or Gleason score of 8 to 10 (28%) tumors. As shown in Table 2, the minor allele frequencies for the 7 polymorphisms in *SOD2* were similar among the 2 cohorts. Three of the SNPs (rs4880, rs2758331, rs2758329) were in linkage disequilibrium with $r^2 \geq 0.8$.

During follow-up in the PHS and HPFS cohorts, there were 77 lethal prostate cancer events, of which 52 were cancer deaths and 25 were distant metastases among living patients. Known prognostic factors, including biopsy Gleason score ($P < .001$), log PSA ($P = .008$), clinical tumor, node, metastases (TNM) stage ($P < .001$), and year of diagnosis ($P < .001$) were associated with lethal prostate cancer. Table 3 shows that 3 of the 7 SNPs were statistically significantly associated with the composite end point of prostate cancer death or metastases among living participants, at $P < .05$. rs6917589 polymorphism was associated with risk of lethal prostate cancer ($P = .006$). Carriage of the C allele in rs4880, which results in the valine to alanine isoform of the enzyme, was associated with a nominally statistically significant decrease in risk of lethal prostate cancer (HR, 0.37 for

Table 1 Patient Characteristics at Time of Diagnosis for the Test and Validation Cohorts

Characteristic	Test Cohort (n = 816)	Validation Cohort (n = 612)
Median Follow-Up, Years	10.2	6.8
Median Age at Time of Treatment (IQR), Years	73 (68-76)	64 (59-70)
Gleason Score, n (%)		
2-6	486 (60)	148 (24)
7	196 (24)	261 (43)
8-10	85 (10)	173 (28)
Unknown	49 (6)	30 (5)
Clinical Stage, n (%)		
T1/T2	744 (91)	458 (75)
T3/T4/N1	50 (6)	23 (4)
Unknown	22 (3)	131 (21)
PSA at Time of Diagnosis (IQR)	7.3 ng/mL (5.4-11.0)	7.7 ng/mL (5.2-15)
Year of Treatment, n (%)		
1982-1991	83 (10)	18 (3)
1992-2001	535 (66)	285 (47)
2002-2010	198 (24)	309 (51)

Abbreviations: IQR = interquartile range; PSA = prostate-specific antigen.

homozygous C/C and HR, 0.84 for T/C genotype; $P = .04$) as compared with the T/T genotype. This borderline association was also observed for the minor allele genotypes among the other 2 tagging SNPs in linkage disequilibrium with rs4880 (rs2758331 and rs2758329).

In the cohort of patients who underwent radical prostatectomy for prostate cancer (n = 1094), the median age at time of prostatectomy was 65 years and the median PSA at time of diagnosis was 6.2 ng/mL (interquartile range [IQR], 4.7-9.7). In this cohort, 711 patients [65%] had Gleason score ≤ 6 and 1028 patients [94%] had clinical T1/2 tumors. With a median follow-up of 12 years, there were 71 occurrences of lethal prostate cancer, of which 43 were from prostate cancer deaths and 28 were from distant metastases among living patients. There was no association between any of the 7 SNPs in *SOD2* and lethal prostate cancer outcome after adjustment for age at radical prostatectomy, clinical TNM stage, log PSA, biopsy Gleason score, year of diagnosis, and cohort (Table 3).

We further examined the association of the 7 SNPs in *SOD2* with prostate cancer recurrence and with development of metastatic disease in a separate higher-risk cohort of prostate cancer patients who underwent RT from the Dana-Farber Cancer Institute (n = 612). The median follow-up time was 6.8 years (range, 2 months-20 years) from the initiation of RT. There were 277 patients who experienced biochemical recurrence, with a median time to biochemical recurrence of 4.5 years (95% CI, 3.9-5.2 years). Distant metastasis was also assessed as an outcome of interest based on a total of 168 patients who developed distant metastases and had a median time to distant metastasis of 11 years (95% CI, 10.4-13.5 years). In adjusted and unadjusted analyses, there was no association between rs6917589, rs4880, or other SNPs in *SOD2* and distant metastasis or biochemical recurrence (Table 4, and Supplemental Table 1 in the online version).

Discussion

In a cohort of patients with predominantly lower-risk prostate cancer who were treated with definitive RT, the *SOD2* rs6917589 was associated with risk of lethal prostate cancer. There were borderline statistically significant associations between rs2758331 and the functional *SOD2* rs4880 polymorphism and lethal prostate cancer in the test cohort. Of note, these 3 *SOD2* polymorphisms were not predictive of cancer-specific outcomes after radical prostatectomy.

The initial finding was not reproduced in a cohort of men with a greater proportion of intermediate-to high-grade Gleason scores, in whom there was no association between any *SOD2* polymorphism and risk of biochemical recurrence or distant metastasis. This study comes after attention has focused on the lack of reproducibility of candidate gene association studies.[18,19] The Radiogenomics: Assessment of Polymorphisms for Predicting the Effects of Radiotherapy (RAPPER) study included 637 patients who received radical prostate radiotherapy and it rigorously assessed the association between toxicity outcomes and 92 SNPs in 46 genes that had been previously reported to be statistically significantly associated with radiation toxicity. The study failed to reproduce any of the findings, but did report borderline statistical significance for the *SOD2* rs4880.[20] The current study benefits from having a total of 1428 patients treated with RT and is the largest study to our knowledge

Table 2 Allelic Variation of the 7 Candidate SNPs in the *SOD2* Gene Among Study Patients in the Test (n = 816) and Validation (n = 612) Cohorts of Men With Prostate Cancer Treated With Radiation

SNP	Major/Minor Allele	MAF (Test), %	MAF (Validation), %	Type	Annotation
rs6917589	A/G	24	24	–	3′ of *SOD2*
rs2758331[a]	G/T	48	47	Synonymous	Intron
rs4880[a]	T/C	49	49	Nonsynonymous (valine/alanine)	Exon 2
rs2758329[a]	A/G	48	48	–	3′ of *SOD2*
rs5746151	G/A	<10	<10	–	3′ of *SOD2*
rs2842980	A/T	21	21	–	3′ of *SOD2*
rs7855	T/C	<10	<10	–	3′ UTR, exon

Abbreviations: A = adenine; C = cytosine; G = guanine; MAF = minor allele frequency; SNP = Single nucleotide polymorphism; T = thymine; UTR = untranslated region.
[a]SNPs are in linkage disequilibrium with each other.

Table 3 Associations Between *SOD2* Polymorphisms and Lethal Prostate Cancer Among Prostate Cancer Patients Undergoing Radiation Therapy or Radical Prostatectomy in the Test Cohort, Adjusted for Gleason Score, PSA, Clinical Stage, Age at Time of Treatment, Year of Diagnosis, and Cohort (PHS or HPFS)

	Radiation Therapy Cohort (n = 816)						Radical Prostatectomy Cohort (n = 1094)					
SNP	Total, n	Events, n	Unadjusted HR (95% CI)	*P*	Adjusted HR (95% CI)	*P*	Total, n	Events, n	Unadjusted HR (95% CI)	*P*	Adjusted HR (95% CI)	*P*
SOD2												
rs6917589												
GG	46	5	1.70 (0.66-4.38)	.06	2.62 (0.98-7.02)	.006	39	2	0.86 (0.21-3.56)	.64	0.78 (0.18-3.25)	.54
AG	230	32	1.80 (1.10-2.94)		2.21 (1.31-3.72)		334	25	1.26 (0.75-2.10)		1.31 (0.76-2.25)	
AA	429	32	REF		REF		598	35	REF		REF	
rs2758331[a]												
TT	177	9	0.52 (0.24-1.17)	.05	0.37 (0.16-0.83)	.04	234	18	1.05 (0.55-2.01)	.66	1.10 (0.56-2.13)	.67
GT	338	46	1.28 (0.74-2.20)		0.84 (0.47-1.50)		498	31	0.82 (0.47- 1.46)		0.84 (0.47-1.52)	
GG	193	18	REF		REF		246	19	REF		REF	
rs4880[a]												
CC	182	9	0.52 (0.23-1.17)	.04	0.37 (0.16-0.84)	.04	254	19	1.02 (0.53-1.94)	.77	1.10 (0.57-2.13)	.67
TC	343	47	1.31 (0.75-2.28)		0.90 (0.50-1.61)		495	32	0.85 (0.48-1.51)		0.85 (0.47-1.53)	
TT	182	17	REF		REF		232	18	REF		REF	
rs2758329[a]												
GG	170	9	0.53 (0.24-1.18)	.05	0.39 (0.17-0.88)	.06	228	18	0.99 (0.52-1.87)	.27	1.08 (0.56-2.09)	.37
AG	336	46	1.27 (0.74-2.19)		0.86 (0.48-1.53)		500	27	0.66 (0.37-1.18)		0.72 (0.40-1.31)	
AA	188	18	REF		REF		236	20	REF		REF	
rs5746151												
GA/AA	86	7	0.75 (0.35-1.65)	.48	0.59 (0.25-1.37)	.22	121	6	0.71 (0.31-1.64)	.42	0.62 (0.27-1.46)	.27
GG	627	63			REF		858	61	REF		REF	
rs2842980												
TT	35	2	0.59 (0.14-2.42)	.69	0.86 (0.20-3.62)	.64	43	3	0.92 (0.29-2.97)	.94	0.78 (0.24-2.53)	.79
AT	224	25	1.09 (0.67-1.78)		1.26 (0.76-2.07)		327	22	0.91 (0.55-1.53)		0.85 (0.50-1.44)	
AA	444	46	REF		REF		604	43	REF		REF	
rs7855												
TC/CC	75	7	0.90 (0.41-1.97)	.80	0.97 (0.44-2.13)	.94	110	6	0.74 (0.32-1.71)	.48	0.45 (0.19-1.06)	.07
TT	639	66	REF		REF		875	64	REF		REF	

Abbreviations: A = adenine; C = cytosine; G = guanine; HR = hazard ratio; REF = reference; SNP = single nucleotide polymorphism; T = thymine.
[a]SNPs in linkage disequilibrium with each other.

Table 4 Associations Between *SOD2* SNPs and Distant Metastases in the Validation Cohort (n = 612), Dana-Farber Cancer Institute Prostate Cancer Patients Who Underwent Radiation Therapy

SNP	Total	Events	Unadjusted HR (95% CI)	*P*	Adjusted HR (95% CI)	*P*
rs6917589				.97		.97
GG	42	13	1.04 (0.59-1.86)		1.04 (0.58-1.88)	
AG	210	51	0.97 (0.69-1.36)		1.05 (0.74-1.48)	
AA	354	100	REF		REF	
rs2758331				.99		.85
TT	128	36	1.02 (0.66-1.57)		0.99 (0.63-1.55)	
GT	296	82	1.03 (0.72-1.48)		1.09 (0.76-1.58)	
GG	180	46	REF		REF	
rs4880				.94		.87
CC	141	38	0.93 (0.60-1.44)		0.93 (0.60-1.46)	
TC	298	82	0.98 (0.68-1.42)		1.04 (0.71-1.52)	
TT	165	44	REF		REF	
rs2758329				.99		.90
GG	136	37	0.97 (0.63-1.50)		0.94 (0.60-1.46)	
AG	296	82	1.00 (0.70-1.44)		1.03 (0.71-1.50)	
AA	173	45	REF		REF	
rs5746151				.18		.14
GA/AA	78	25	1.34 (0.87-2.06)		1.40 (0.89-2.20)	
GG	528	139	REF		REF	
rs2842980				.54		.33
AT/TT	238	63	0.91 (0.66-1.24)		0.85 (0.61-1.18)	
AA	367	101	REF		REF	
rs7855				.63		.14
TC/CC	72	19	0.89 (0.55-1.44)		0.69 (0.41-1.14)	
TT	534	145	REF		REF	

Abbreviations: A = adenine; C = cytosine; G = guanine; HR = hazard ratio; REF = reference; SNP = single nucleotide polymorphism; T = thymine.

to investigate the relationship between candidate gene polymorphisms and prostate cancer outcome after RT.

Although it is possible that our initial observations of statistically significant associations for *SOD2* SNPs and outcomes were due to chance, it is also possible that differences in the study population, follow-up times, available outcomes, and clinical variables might also account for the lack of consistent results in the validation cohort. For example, patients in the validation cohort tended to be younger and to have more intermediate-risk disease than the older, predominantly low-risk patients in the test cohort. Also, the test cohort had substantially longer follow-up than the validation cohort. Androgen deprivation therapy was also commonly used in the validation cohort and is estimated to have been used much less often in the test cohort. The end points were also different. The test cohort used lethal prostate cancer as the outcome, with most events being death from prostate cancer. Distant failure was not used as a separate end point in the first cohort because of the low number of verified self-reported events, which was potentially due to less use and availability of posttreatment PSA monitoring or radiographic imaging to detect distant metastases compared with the more modern validation cohort. In the validation cohort, biochemical recurrence and distant metastasis were validated via medical records and were deemed the most appropriate because few deaths from cancer had occurred by the end of follow-up. Data were not available for local recurrence after RT because of a lack of consistent screening and reporting of local recurrence in the test cohort. Last, many of the prostate biopsies from the test cohort were assigned a Gleason score during an earlier time period than the DFCI cohort. We previously reported that there is an upgrading in Gleason score after modern standardized review of the original biopsy specimens from these cohorts,[21] making it challenging to compare the distribution of Gleason scores across the test and validation cohorts.

The *SOD2* rs4880 T/C polymorphism has been well studied and postulated to result in increased ability to neutralize ROS because of more efficient uptake into the mitochondrial matrix.[22] It has been associated with aggressive prostate cancer incidence among men with low antioxidant nutritional intake.[23,24] However, there are conflicting data regarding the association between rs4880 and toxicity after RT. Some studies identified an association of rs4880 with increased risk of subcutaneous fibrosis in breast cancer patients who underwent RT[11] and with Grade $\geq$ 3 side effects in predominantly breast cancer and head and neck cancer populations.[13] Another study by Green et al refuted the association between *SOD2* and radiotherapy complications in breast cancer patients.[14] Our study did not find a reproducible association between 7 of the *SOD2* SNPs and prostate cancer outcomes, but there was a suggestion of

increased survival after RT for the rs4880 polymorphism and of decreased survival after RT for the rs6917589 polymorphism.

Because the interaction between SOD2 and the tumor microenvironment is more complex than a single enzymatic reaction, pathway analysis of SNPs might yet detect clinically significant associations by taking into account other key enzymes involved in regulating oxidative stress. For example, as shown in Figure 1, after SOD2 catalyzes the conversion of superoxide anion to hydrogen peroxide, the myeloperoxidase enzyme catalyzes the conversion of hydrogen peroxide to hydrochlorous acid, which is another oxidizing agent that might cause a net effect of increased ROS. Alternatively, CAT and GPX might catalyze conversion of hydrogen peroxide to neutral species. Therefore, the overall effect of *SOD2* polymorphisms might be dependent on the activity of myeloperoxidase, CAT, GPX, and other factors that alter the local ROS concentration. The model might also need to take the nutritional status of the patient into account, because our collaborative group has previously reported an interaction between antioxidant status, such as plasma selenium, and a *SOD* polymorphism as related to the incidence of aggressive prostate cancer.[23,25]

The present study benefits from a large sample size and 2 diverse cohorts to independently assess the association between *SOD2* polymorphisms and prostate cancer outcome after RT. Moreover, in the test cohort, we were able to make comparisons with men who underwent radical prostatectomy. A limitation of the study is that we examined only germline polymorphisms and therefore could not assess the genetic changes within the tumor that might affect tolerance to oxidative stress. We also were not able to directly measure the degree of ROS within the tumor or stroma. Last, a pathway analysis might improve the ability to determine the complex interaction between *SOD2* polymorphisms and other genes involved in regulating antioxidant stress.

Conclusion

The present study showed that the most common germline polymorphisms in the *SOD2* are unlikely to have a clinically significant effect on all patient outcomes after RT when treated individually. Although not validated, genetic variants in *SOD2* might have an effect that is specific to low-risk prostate cancer patients, and merits further study.

Clinical Practice Points

- Germline polymorphisms in *SOD2* might modulate the effect of RT by altering local reactive oxygen species.
- In this study we examined the predictive effect of germline polymorphisms in *SOD2*, including the functional rs4880 variant, on lethal prostate cancer after treatment with RT.
- There was a significant association between *SOD2* polymorphisms and lethal prostate cancer.
- This finding was not validated in a separate cohort with different clinical characteristics but might be specific to a lower-risk population.
- Results of this study suggest that previous in vitro findings linking SOD2 activity to radiation response might be relevant in the clinical setting as a predictive biomarker of response to RT.
- The finding remains to be validated in a low-risk cohort.

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Disclosure

The authors have stated that they have no conflicts of interest.

Supplemental Data

The supplemental table accompanying this article can be found in the online version at http://dx.doi.org/10.1016/j.clgc.2014.12.018.

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Supplemental Table 1 **Associations Between *SOD2* SNPs and Biochemical Recurrence in the Validation Cohort (n = 612), Dana-Farber Cancer Institute Prostate Cancer Patients Who Underwent Radiation Therapy**

SNP	Total	Events	Adjusted HR (95% CI)	*P*
rs6917589				.84
GG	33	18	0.91 (0.55-1.49)	
AG	185	98	1.05 (0.81-1.36)	
AA	306	158	REF	
rs2758331				.63
TT	114	60	1.13 (0.80-1.59)	
GT	260	134	0.97 (0.73-1.30)	
GG	148	80	REF	
rs4880				.61
CC	125	64	1.16 (0.82-1.63)	
TC	263	136	1.00 (0.74-1.35)	
TT	134	73	REF	
rs2758329				.54
GG	121	63	1.14 (0.81-1.61)	
AG	259	132	0.96 (0.72-1.29)	
AA	143	78	REF	
rs5746151				.08
GA/AA	69	42	1.36 (0.96-1.94)	
GG	455	232	REF	
rss2842980				.13
AT/TT	201	102	0.82 (0.64-1.06)	
AA	322	172	REF	
rs7855				.22
TC/CC	61	33	0.79 (0.54-1.15)	
TT	463	241	REF	

Abbreviations: A = adenine; C = cytosine; G = guanine; HR = hazard ratio; REF = reference; SNP = single nucleotide polymorphism; T = thymine.

Dissecting the Dual Role of AMPK in Cancer: from Experimental to Human Studies

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Running Title: The Dual Role of AMPK in Cancer

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Abstract

The precise role of 5'AMP-activated kinase (AMPK) in cancer and its potential as a therapeutic target is controversial. While it is well established that activation of this energy sensor inhibits the main anabolic processes that sustain cancer cell proliferation and growth, AMPK activation can confer on cancer cells the plasticity to survive under metabolic stress such as hypoxia and glucose deprivation, which are commonly observed in fast growing tumors. Thus, AMPK is referred to as both a "conditional" tumor suppressor and "contextual" oncogene. To add a further layer of complexity, AMPK activation in human cancer tissues and its correlation with tumor aggressiveness and progression appears to vary in different contexts. The current review discusses the different faces of this metabolic regulator, the therapeutic implications of its modulation and provides an overview of the most relevant data available on AMPK activation and AMPK activating drugs in human studies.

Introduction

5' AMP-activated kinase (AMPK) is a central metabolic sensor that stands at the crossroad between metabolic and signaling networks. In 2003, the discovery of the tumor suppressor liver kinase B1 (LKB1) as the major upstream kinase of AMPK established a link between an energy regulator and cancer pathogenesis, suggesting that the tumor suppressor functions of LKB1 could be mediated by AMPK (1-3). Since then, *in vitro* and *in vivo* studies have been conducted to dissect the role of AMPK in cancer initiation and progression, using AMPK modulating drugs. The functional consequences of AMPK activation in cancer appear to be much more complex than initially thought and AMPK can behave as both cancer "friend" or "foe" in a context-specific manner.

Drug-induced supra-physiological activation of AMPK reduces tumor growth *in vitro* and in pre-clinical models through the suppression of key biosynthetic pathways (reviewed in (4, 5)). However, physiological activation of AMPK in response to a broad range of stresses (e.g. hypoxia, glucose deprivation, and matrix detachment) provide cancer cells with the flexibility to adapt and survive metabolic stress (metabolic adaptation) (reviewed in (6)). Immunohistochemical evaluation of AMPK status in human tissues has revealed that the levels of AMPK activation are heterogeneous in different tumor types, while discordant data have been reported on the correlation between AMPK activation and tumor prognosis.

Here, we discuss the "two faces" of AMPK, the therapeutic benefit of AMPK modulators and we review the current data available on AMPK activation and AMPK activating drugs in human studies. Throughout the review, we will associate AMPK with both the terms "tumor promoter" and "tumor suppressor". However, we do not intend to define AMPK as a classical *bona fide* tumor suppressor gene such as LKB1, which is mutated or deleted in several cancers, rather to emphasize the fact that AMPK activation may result in tumor growth inhibition, cell cycle arrest, and apoptosis of cancer cells in some tumor types/contexts. Interrogating the cBioPortal data, the frequency of mutation/deletion in the genes

codifying for AMPK catalytic subunits α1 (*PRKAA1*) and α2 (*PRKAA2*) ranges from 0.2-3.4% and from 0.2-10.3%, respectively (7).

AMPK: a unique metabolic "guardian" with pleiotropic downstream targets

AMPK is a heterotrimeric Ser/Thr kinase complex characterized by a catalytic α subunit and two regulatory subunits (β, γ), which exist in different isoforms making up to 12 different heterotrimers. The different subunits show tissue-specificity and may contribute to tumor cell growth and proliferation independently (8-10). The γ subunit contains four-tandem sequence repeats known as CBS repeats, which functions as four adenine nucleotide-binding domains. Site 2 is always unoccupied, site 4 is permanently bound by AMP, whereas sites 1 and 3 can be competitively bound by either AMP, or ADP, or ATP (11, 12).

AMPK functions as an energy sensor to restore energy homeostasis at cell and organismal levels in conditions of metabolic stress that reduce ATP levels either by inhibiting its production (e.g. hypoxia, glucose deprivation, and treatment with biguanides drugs or xenobiotics) or by accelerating its consumption (e.g. muscle contraction), resulting in increased ADP and AMP levels. For a detailed description of AMPK regulation, we refer readers to other excellent reviews (13, 14). However, a brief description of the biochemical circuits regulating AMPK follows. The binding of ADP and/or AMP to the γ subunit both promotes phosphorylation by upstream kinases and inhibits dephosphorylation of the residue Thr172 within the activation loop of the catalytic domain, which is required for the full activity of the kinase. Furthermore, the binding of AMP (but not ADP) causes a further allosteric activation of the phosphorylated kinase. The two major upstream kinases responsible for AMPK activation are the tumor suppressor LKB1 and Ca2+/calmodulin-dependent protein kinase kinase 2 (CaMKK2). An activating role, still not well characterized, for the transforming growth factor beta-activated kinase 1 (TAK1) has also been described. LKB1 activates AMPK during energy stress, whereas CaMKK2 activity is induced by increased intracellular Ca2+ levels, regardless of the energy status of the cells

(reviewed in (13)). However, CaMKK2 can compensate for the absence of LKB1 in mediating AMPK phosphorylation (15). In addition to AMP, ADP and Ca2+, recent studies have also identified reactive oxygen species (ROS) as additional upstream activators of AMPK, acting in an LKB1-independent manner (16) (Fig. 1). Once activated, AMPK maintains energy balance by switching off anabolic pathways that consume ATP and NADPH, while switching on catabolic pathways that generate ATP both by direct phosphorylation of metabolic enzymes, and through longer-term effects mediated by phosphorylation of transcription factors and co-activators (14). Thus AMPK can restrain cell growth by: (i) inhibiting protein synthesis [through direct phosphorylation of mammalian target of rapamycin complex 1 (mTORC1) signaling members tuberous sclerosis complex 2 (TSC2) and Raptor], (ii) blocking fatty acid (FA) and cholesterol biosynthesis [through direct phosphorylation of the enzymes acetyl-CoA carboxylase 1 (ACC1) and 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR) and inhibition of the lipogenic transcription factors sterol regulatory element-binding proteins (SREBPs) and carbohydrate-responsive element-binding protein (ChREBP)], required for new membrane formation in proliferating cells, (iii) inducing cell cycle arrest and apoptosis [through several mechanisms including stabilization of p53, regulation of the cyclin-dependent kinase inhibitors $p21^{Waf1}$ and $p27^{Cip1}$, phosphorylation of the hippo signaling member angiomotin-like 1 (AMOTL1), an upstream inhibitor of Yes-associated protein (YAP) (13, 17, 18)], while promoting cell survival mechanisms during metabolic stress (19), as discussed below (Fig. 2).

Role of AMPK in cancer: pre-clinical studies

AMPK as a tumor suppressor

Since the role of LKB1 as tumor suppressor was well established, AMPK was primarily considered as a component of the LKB1-mediated tumor suppressor cascade and much less was known regarding its own independent role in cancer. This was due to the fact that most of the data were generated utilizing the AMPK activators AICAR and metformin, which also display AMPK-

independent mechanisms or by experimental evidence in models of LKB1 inactivation, which affect an additional 12 AMPK-related downstream kinases, beyond AMPK. The role of the AMPK-related kinases is still not very well characterized, though they might themselves contribute to the tumor suppressive functions of LKB1, as well as have independent functions (20). Experiments of genetic ablation of AMPK, the use of direct AMPK activators, and detailed phosphorylation studies in different cancer models have recently helped to address this issue. Faubert et al. have reported that the ubiquitous knockout (KO) of AMPKα1, the only catalytic subunit expressed in B cells, accelerates the development of lymphomas in transgenic mice overexpressing c-Myc, suggesting that AMPK loss can cooperate with oncogenic drivers to promote tumorigenesis in a tissue-specific manner. The underpinning mechanism for AMPK tumor suppressor activity is the ability of the kinase to exert an "anti-Warburg" effect by downregulating hypoxia-inducible factor 1-alpha (HIF-1α) and its downstream glycolytic genes, which conversely are upregulated in AMPKα1 KO mice (21).

Aside from antagonizing the Warburg effect, AMPK has also been shown to exert its ''metabolic'' tumor-suppressor role by inhibiting unchecked mTORC1 activity and *de novo* lipogenesis, required both during G1/S and G2/M phases. We have recently observed increased *de novo* fatty acid (FA) synthesis concomitant to reduced AMPK activation and phosphorylation of its major target ACC1 (the rate-limiting enzyme for FA synthesis), prior to cytokinesis initiation. In this view, by inhibiting *de novo* FA synthesis and FA incorporation into membranes, activation of AMPK would prevent cells from completing mitosis, arresting them at a "lipogenic" G2/M checkpoint. This was indeed observed under direct supra-physiological activation of AMPK (22). Cell cycle arrest (via decreased fraction of cells in the S phase) and/or apoptosis, was previously confirmed using ACC1 and fatty acid synthase (FASN) siRNA to directly inhibit FA synthesis (23, 24).

AMPK also plays a direct metabolic-independent role in cell cycle regulation (25-27). A fine-tuned biphasic activation of AMPK has been shown to be required for proper mitotic progression (28).

However, alteration of the dynamic spatial and temporal regulation of AMPK by either its sustained activation or depletion can result in microtubule misalignment, spindle misorientation, abnormal chromosome segregation followed by mitotic catastrophe and polyploidy (e.g. observed under metformin treatment) or mitotic delay (e.g. observed in AMPK-silenced cells) (27, 29). Thus, cell cycle arrest induced by persistent supra-physiological activation of AMPK could be ascribed to both the inhibition of *de novo* FA synthesis (metabolic role) as well as mitotic spindle assembly/chromosome segregation abnormalities (non-metabolic role). Recently, a role for the subunit AMPK α1 in the direct regulation of cell cycle, independently of energy balance, has also emerged (30).

A third mechanism in favor of AMPK's behavior as a "tumor suppressor" has been described by Shen et al., showing AMPK-dependent phosphorylation of the oncogene BRAF at Ser729. This phosphorylation prevents BRAF interaction with the scaffolding protein kinase suppressor of Ras 1 (KSR1), leading to the suppression of the oncogenic MEK-ERK signaling and consequent impairment of cell proliferation and cell cycle progression (31).

Furthermore, additional mechanisms of action to suppress tumor growth have been proposed. Chou et al. showed that AMPK knock down promotes "epithelial-mesenchymal transition" (EMT) in breast and prostate cancer cell lines by reducing the expression of forkhead box O3 (Foxo3a) and E-cadherin in conjunction with increased expression of vimentin, Y-box-binding protein-1 (YB-1), Snail, and the formation of F-actin stress fibers (32). These results suggested that AMPK activation counteracts EMT, the process through which epithelial cells are thought to acquire cancer stem cell-like properties and gain the ability to breach basement membranes and metastasize to distant sites. DeRan et al. showed that AMPK activation induces phosphorylation of the hippo signaling component AMOTL1, which results in the cytoplasmic sequestration and inhibition of YAP and its targeted genes, involved in proliferation and survival. This mechanism was abolished when AMPK expression was silenced, suggesting that loss of AMPK activity may contribute to tumorigenesis through AMOTL1

destabilization, leading to hyperactivation of YAP (18). Finally, AMPK may be inactivated by its ubiquitination and degradation by the cancer-specific MAGE-A3/6-TRIM28 ubiquitin ligase. MAGE-A3 and MAGE-A6 proteins, normally expressed only in the male germline, are frequently re-activated in human cancers, they are necessary for cancer cell viability, and sufficient to induce cell transformation. Screening for targets of MAGE-A3/6-TRIM28 complex revealed that it ubiquitinates and degrades AMPKα1, leading to inhibition of autophagy, activation of mTORC1 signaling, and hypersensitization to AMPK agonists, such as metformin. These findings elucidated a germline mechanism commonly hijacked in cancer to suppress AMPK (33).

Further evidence also supports the tumor suppressor role of AMPK in some tumor types and genetic contexts. First, protein kinase B (Akt), has been reported to induce AMPK phosphorylation at Ser485, reducing its activation by LKB1 (34). This might occur in tumors in which Akt is hyperactivated due to phosphatase and tensin homolog (PTEN) loss-of-function mutations, or activating mutations in phosphoinositide-3-kinase (PI3K). Second, AMPK activation is suppressed in melanoma cells carrying the most common BRAF mutation V600E, which induces a constitutively active downstream ERK. The lack of AMPK activity is due to ERK and ribosomal S6 kinase (RSK)-mediated phosphorylation of LKB1, which prevents its binding/activation of AMPK. These data suggested that suppression of LKB1/AMPK pathway might play an important role in BRAF V600E-driven tumorigenesis (35). Third, inhibition of AMPK has been observed in a PTEN-deficient model of thyroid cancer and in NSCLC cells expressing the mitochondrial heat shock protein 90 chaperone TRAP-1 (36). Fourth, in fumarate hydratase-deficient kidney tumors and cell lines from patients with hereditary leiomyomatosis renal cell cancer (HLRCC), which are characterized by a metabolic shift to aerobic glycolysis, AMPK levels are decreased. AMPK reduction leads to diminished expression of the DMT1 iron transporter, cytosolic iron deficiency, and activation of the iron regulatory proteins, IRP1 and IRP2, resulting in increased expression of HIF-1α. Silencing of HIF-1α or activation of AMPK diminishes

invasive activities of the HLRCC cell line UOK262, indicating that overexpression of HIF-1α and downregulation of AMPK contribute to the oncogenic growth of fumarate hydratase- deficient cells (37). Recently, a study from Rodriguez et al. showed that Cytochrome P450-1A1, constitutively expressed in the majority of breast cancer tumors, promotes breast cancer proliferation and survival, at least in part, through suppression of AMPK signaling (38). Finally, reduced expression of the catalytic α2 subunit has been reported in some cases of hepatocellular carcinomas and it is associated with enhanced tumor cell growth in mouse xenografts (10).

Taken together, these results suggest that in specific genetic, metabolic, and signaling contexts, AMPK can exert a tumor suppressor role (Fig. 3).

AMPK as contextual tumor promoter

The ability to survive in conditions of metabolic stress, such as hypoxia/nutrient deprivation, or matrix detachment is fundamental to cancer cells. Several mechanisms by which the AMPK pathway supports this plasticity have been described. These include: (i) the induction of autophagy by AMPK-dependent phosphorylation of the unc-51-like kinases (ULK) (39), (ii) the promotion of FA oxidation (FAO) to generate ATP (40, 41), (iii) transcriptional changes induced by phosphorylation of the core histone H2B (42), (iv) the increase of intracellular NADPH levels through the activation of FAO/inhibition of FA synthesis to neutralize cytotoxic ROS (43) (Fig. 3). Intriguingly, while in nutrient-replete conditions, the AMPK energy-sensing pathway and the PI3K/Akt cascade converge on mTOR with opposing regulatory effects, under glucose depletion, both AMPK and Akt are activated and coordinately support cell survival (44). Thus, whereas the LKB1/AMPK pathway can act as a tumor suppressor through its ability to restrain tumor growth, it can also behave as "tumor promoter", allowing tumor cells to be more resistant to metabolic stress, such as when tumor growth exceeds the capacity of its blood supply to deliver oxygen and nutrients (Fig. 4). Recent experimental evidence *in vitro,* using the direct AMPK activator A-769662, indeed supports this notion (45). AMPK activation can also

promote tumor growth in specific tumor types and genetic contexts, even in nutrient-replete conditions. Recent evidence showed the key role of AMPK in supporting tumor growth in aggressive breast and astrocytic tumors (46-49). Moreover, in contrast to the results obtained by Faubert et al in a lymphoma model (21), MYC has been shown to establish a dependence on AMPK-related kinase 5 (ARK5) to maintain metabolic homeostasis and cell survival. Depletion of ARK5 prolongs survival in MYC-driven mouse models of hepatocellular carcinoma, suggesting that targeting cellular energy homeostasis is a valid therapeutic strategy to eliminate tumor cells that express deregulated MYC (50).

The therapeutic benefit of AMPK modulators: the metformin paradox

The better understanding of the dichotomous role of AMPK in cancer has also brought about the careful re-evaluation of the use of AMPK modulators in cancer therapy. In this regard, the case of metformin is emblematic.

The interest in using AMPK activators began as evidence was accumulating for the anti-tumorigenic role of the LKB1/AMPK axis. The anti-proliferative and growth-suppressing effects of supra-physiological activation of AMPK have been shown *in vitro* and in pre-clinical models. Activation was achieved with natural compounds, the AMP mimetic drug AICAR as well as the biguanides metformin and phenformin, which inhibit complex I of the mitochondrial electron transport chain, leading to increased levels of intracellular ADP, AMP, and energy stress (reviewed in (4, 14, 51)) Metformin has received particular attention since it is a safe medication, used as first choice in the treatment of type II diabetes and has been associated with reduced cancer incidence in diabetic patients (52). Thus, it is currently being tested for cancer treatment/prevention in several clinical trials, as discussed below. However, ascribing metformin's anti-tumor properties *in vivo* to AMPK activation has been criticized since the major effect of the drug is the inhibition of hepatic gluconeogenesis, resulting in reduced circulating levels of glucose and insulin, two well-known promoters of tumor cell proliferation. This is also valid for metformin's anti-tumor effects *in vitro*, where several AMPK-

independent mechanisms have been described (45, 53-56). Moreover, the discovery of the so-called "biguanide paradox" has recently suggested that, in specific contexts, metformin-mediated suppression of tumor growth does not depend on AMPK activation but, rather, on its down-regulation. Because cells with a defective LKB1/AMPK pathway are less able to restore ATP levels in response to metabolic stress induced by metformin treatment, LKB1/AMPK-deficient cancer cells are more susceptible to cell death than their counterparts with a functional LKB1/AMPK axis (Fig. 5). Several *in vitro* and *in vivo* studies using metformin, phenformin, or other compounds that cause metabolic stress (AICAR, salicylate, and 2-deoxyglucose) have supported this mechanism (discussed in (57, 58)). In light of this, the use of biguanides may be most effective in combination with agents that inhibit, rather than activate, AMPK and, overall, these data suggest that the use of AMPK inhibitors rather than activators would preferentially trigger cancer cell death in the context of metabolic stress. Interestingly, the chemotherapeutic agent sunitinib has been shown to inhibit AMPK, suggesting that combinatorial treatment of sunitinib and metformin could be clinically relevant (59).

Novel direct AMPK activators have been developed to overcome the off-target effects of metformin and AICAR treatment. The direct activator A-769662 (which binds the β1 subunit) delays tumor formation in PTEN null/LKB1 hypomorphic mice (60). The same compound has been shown to suppress the proliferation of breast, colon, and prostate cancer cells (61-63). A-769662 was however ineffective in models of glioma (56). OSU-53, a direct activator that binds the auto-inhibitory domain of AMPK, displays tumor growth inhibition *in vitro* and *in vivo* in triple-negative breast cancer models (64). The same group reported that AMPK activation by OSU-53 blocks "EMT" in breast and prostate cancer cells by activating Foxo3a, which results in the inhibition of invasive phenotypes *in vitro* and metastatic properties *in vivo* (32). Direct supra-physiological activation of AMPK in nutrient-replete conditions has been also shown to suppress prostate cancer cells growth, in association with mitotic arrest and apoptosis, and to potentiate the effect of anti-androgens *in vitro* (65). The inhibitory effect of

AMPK activation on the androgen receptor (AR) axis at both transcriptional and post-translational levels was previously observed when a supra-physiological activation of AMPK was achieved by treatment with metformin or AICAR (66, 67). Finally, Compound 1, a novel AMPK activator, induces a significant antitumor activity *in vitro* and tumor growth delay in a mouse xenograft model of colorectal cancer (68). The mechanism through which Compound 1 activates AMPK, is however, still uncharacterized.

Taken together, the induction of a persistent, supra-physiological activation of AMPK results in tumor suppression in some cancer types (Fig. 3).

Salicylate, the active metabolite of aspirin following absorption from the gut, was recently identified as a direct AMPK activator, which binds to the same site on the β1 subunit as A-769662 (69). This suggests that AMPK activation might be involved in mediating aspirin's protective effects against cancer. Future pre-clinical studies in genetically engineered AMPK models are however required to validate this hypothesis.

Overall, these apparently conflicting data suggest that both AMPK activators and inhibitors can provide therapeutic benefit in different tumor types, different genetic/metabolic contexts, and different microenvironment conditions. Thus, the choice of AMPK modulators may be different at various phases of tumorigenesis/tumor progression.

AMPK role in cancer: Human studies

AMPK activation in human cancers

Evaluation of AMPK activation in human tissues is not trivial. Early studies have demonstrated that when tissues and organs are removed by dissection at ambient temperature rather than by freeze clamping, ACC phosphorylation both occurred as a post-mortem artifact. Dissection at ambient temperature leads to elevation of AMP and depletion of ATP, presumably due to hypoxia following interruption of the blood supply, resulting in AMPK activation. Moreover, ACC phosphorylation in

tissues such as liver has also been shown to follow a diurnal rhythm and to be influenced by dietary behavior (70). Therefore, analysis of AMPK activity and ACC phosphorylation in human tissues should be interpreted with caution.

AMPK activation has been investigated in fresh frozen and archival tumor tissue from numerous cancer sites, including prostate (63, 71, 72), breast (73, 74), head and neck (75), colorectal (76, 77), gastric (78, 79), liver (80), lung (81-83), ovary (84), and kidney (85, 86). Table 1 summarizes the population-based studies of AMPK activation, measured by protein expression of phosphorylated AMPKα1 (p-AMPKα1, n=16 studies) or its phosphorylated substrate ACC (p-ACC, n=6 studies), with cancer prognosis and clinicopathologic features. Of the 13 studies reporting on p-AMPKα1 at Thr172 and overall, cancer-specific, or progression-free survival, 8 studies found that AMPK activation was associated with improved prognosis among head and neck (75), colorectal (76, 77), gastric (79), liver (80), lung (81), and kidney (85, 86) cancer patients either within the entire study population or within subgroups. Consistent with the findings for p-AMPKα1 at Thr172, one additional study of lung cancer found that higher expression of p-AMPKα1 at Ser485, which inhibits AMPK signaling (14), was associated with shorter survival (82). Conversely, two studies in gastric cancer (78) and in prostate cancer (72) reported associations between higher p-AMPKα1 and disease recurrence; however, the gastric cancer study population was substantially smaller than that of Kim et al. (79). Three additional studies in lung (83) and breast cancer patients (73, 74) found no association between p-AMPKα1 expression and overall survival. In cross-sectional analyses, higher p-AMPKα1 expression was associated with lower tumor grade and/or stage in breast (73), head and neck (75), colorectal (76), gastric (79), liver (80), and ovarian (84) cancer, while 4 additional studies in prostate (72), breast (74), gastric (78), and lung (81) cancer found no associations with clinicopathologic features. In contrast, Choudhury et al. found increasing p-AMPKα1 expression with higher tumor grade in prostate cancer

specimens (63). Overall, these human studies support the hypothesis that AMPK activation may delay disease progression in several cancer types.

Of the 6 studies that used protein expression of p-ACC at Ser79 to characterize AMPK activation, higher p-ACC was associated with worse overall survival (82) and disease recurrence (83) among lung cancer patients, and with worse overall survival among head and neck cancer (75) and kidney cancer(86) patients. In contrast, higher p-ACC was associated with improved overall survival and progression-free survival in colorectal cancer patients (77). Lastly, no correlation was observed between p-ACC expression and Gleason grade in prostate tumors (71). A better understanding of the effects of ACC inactivation and its downstream targets in different tumor tissues will help elucidate the complex role of AMPK activation in carcinogenesis.

Tumor expression of specific AMPK α, β, and γ subunits in relation to cancer outcomes has been explored in patients with melanoma (87), kidney cancer (85, 86), breast cancer (74), cervical cancer (88), lymphoma (89), ovarian cancer (84, 90, 91), lung cancer (82), and colorectal cancer (92). Total AMPKα1 protein expression, which captures both phosphorylated and non-phosphorylated AMPKα1, was associated with improved overall and disease-specific survival among 128 melanoma patients (87). Total AMPKα1/α2 protein expression was associated with improved progression-free survival ($p=0.04$) and borderline associated with overall survival ($p=0.06$) in 37 renal cell carcinoma patients (85). Using publicly available data from the Cancer Genome Atlas (TCGA), overexpression of the genes encoding for AMPKα1, α2, β1, β2, and γ1 subunits were also associated with improved overall survival ($p\leq0.05$) in 417 clear cell renal cell carcinoma patients (86). In a discovery (n=166) and validation (n=609) cohort of breast cancer patients, total AMPKα expression was associated with longer relapse-free ($p=0.016$ and $p=0.06$, respectively) and breast cancer-specific ($p<0.001$ and $p=0.005$, respectively) survival (74). Using fluorescence *in situ* hybridization, amplification of the gene encoding AMPKα1 was not significantly associated with lymph node positivity ($p=0.085$) in pretreatment cervical biopsies among

31 cervical cancer patients (88). Using the Oncomine database, Hoffman et al. reported an association between higher expression of the genes encoding the regulatory AMPKβ1 and β2 subunits and increased 5-year survival (p=0.001 and 0.021, respectively) among diffuse large B cell lymphoma patients; marginal associations were found for higher expression of the gene encoding AMPKα1 and improved survival (p=0.0751), and higher expression of the gene encoding AMPKγ3 and worse survival (p=0.0646) (89). Similarly in a series of 70 ovarian cancer patients, higher protein expression of p-AMPKβ1 at Ser182 was associated with lower tumor grade (n=70, p=0.009) and improved overall survival in the subgroup of patients with serous subtype (n=46, p=0.037) and advanced-stage disease (n=54, p=0.0016) (90). Phosphorylation of AMPKβ1 at Ser182 has not been shown to affect the kinase activity, but is associated with nuclear localization (93). Another study of total AMPKβ1 in ovarian cancer also found that higher protein expression was associated with early tumor stage (p=0.008), lower tumor grade (p=0.013), and absence of metastasis (p=0.008) (84). This same research group previously demonstrated that higher expression of the gene encoding AMPKα2, measured by quantitative PCR, was associated with improved overall (p=0.030) and disease-free (p=0.014) survival in a hospital-based series of 76 ovarian cancer patients, though gene expression of the α1, β1, β2, γ1, and γ2 subunits were not associated with outcomes (91). Zupa et al., in addition to the findings for p-AMPKα1 and p-ACC listed in Table 1, reported an association between higher protein expression of p-AMPKβ1 at Ser108, indicative of AMPK activation (93), and short- *vs.* long-term survival (p=0.0286) among 28 pathologic stage N0 non-small-cell lung cancer patients (82). Lastly, Vetvik et al. found that tumor expression of the gene encoding AMPKβ1 was positively correlated with advanced tumor stage, but not with the number of affected lymph nodes, in specimens from 60 colorectal cancer patients (92).

With the exception of Zupa et al. and Vetvik at al., these studies suggest that higher tumor expression of specific AMPK subunits may be related to favorable clinicopathologic features and improved outcomes

among cancer patients. Additional studies are warranted to confirm these findings in larger study populations and across cancer sites.

Differential expression of AMPK/ACC in tumor *vs.* normal tissue has been reported in a few neoplasms, including liver (80), ovarian (90, 91), thyroid (94), cervical (95), brain (47), skin (87), prostate (63, 71, 72, 96), and colorectal cancer (92). In hepatocellular carcinoma, protein expression of p-AMPKα1 at Thr172 was downregulated in 62% of tumor *vs*. distant normal liver tissue (80). In ovarian specimens, protein expression of p-AMPKβ1 at Ser182 was significantly higher ($p=0.038$) in carcinoma compared to borderline tumors and normal ovaries (90). Li et al. also found higher expression of the genes encoding AMPKα2, β1, β2, γ1, and γ2 ($p\leq0.001$), but not AMPKα1 ($p=0.320$), in primary cancer *vs.* normal ovarian tissue (91). In papillary thyroid carcinoma patients, protein expression of total AMPKα, p-AMPKα1 at Thr172, and p-ACC at Ser79 was elevated ($p<0.001$) in carcinoma *vs.* paired non-neoplastic tissue (94). Similarly, protein expression of AMPKα1 was significantly higher ($p<0.001$) in tumor *vs.* normal epithelium in cervical cancer patients (95). In a small study of brain cancer, high protein expression of p-ACC at Ser79 was seen in all glioblastoma specimens compared to absence of expression in normal brain (47). In melanoma patients, total AMPKα1 protein expression was increased in primary melanoma *vs.* dysplastic nevi ($p<0.005$), but slightly decreased in metastatic vs. primary melanoma specimens ($p<0.05$) (87). In prostate cancer patients, both p-AMPKα1 at Thr172 and p-ACC at Ser79 were expressed in tumor tissue, compared to no detectable expression in non-paired benign prostate hyperplasia samples (63). Two additional prostate studies reported elevated expression of p-AMPKα1 at Thr172 and p-ACC at Ser79 ($p<0.001$) in prostate tumor *vs.* non-neoplastic tissue (71, 72). Utilizing the Oncomine database, the gene encoding AMPKβ1 was expressed at greater levels in metastatic *vs.* primary prostate cancer in publicly available data from 4 studies (96). Lastly, expression of the gene encoding AMPKβ1 was significantly higher in colorectal cancer *vs.* adjacent mucosa (92). Taken together, these studies support that AMPK dysregulation contributes to neoplastic transformation.

In summary, AMPK expression/activation varies by tumor stage and histology, clinical outcomes, and tissue type (normal, tumor, metastatic). Most of the studies in tumor tissue support a role of AMPK activation, measured by phosphorylation at Thr172, in delaying tumor progression. However, comparing tumor to non-neoplastic tissue suggests that AMPK may be involved in tumor initiation. Thus, evidence from human studies also underscores the dual role of AMPK in carcinogenesis.

AMPK-activating drugs in humans: metformin, phenformin, and aspirin

Several review articles and meta-analyses on metformin and cancer risk have been published in recent years. A 2012 meta-analysis of randomized controlled trials among participants with or at risk of type 2 diabetes did not find reduced cancer incidence for treatment with metformin *vs.* placebo/usual care or active comparators (n=9 studies; summary relative risk (RR): 1.02; 95% confidence interval (CI): 0.82-1.26) (97). Meta-analyses of observational studies among diabetics have shown a reduced risk of cancer associated with metformin use: the fixed-effect summary RRs [95% CI] were 0.70 [0.67-0.73] for 9 cohort studies (98), 0.90 [0.84-0.98] for 13 case-control studies (98), and 0.73 [0.61-0.88] for 21 cohort and case-control studies combined (99). However, both meta-analyses exhibited significant between-study heterogeneity, with Thakkar et al. reporting random-effects model estimates that were attenuated (summary RR: 0.85; 95% CI: 0.65-1.11) among cohort studies, but retained significance (summary RR: 0.71; 95% CI: 0.57-0.88) among case-control studies (98). Inconsistent results may be due to variations in metformin dose, duration of metformin use, length of follow-up, type of comparison group (diabetics taking non-metformin anti-diabetic medications, diabetics on alternative therapy, or non-diabetics), outcome assessed (incident cancer or cancer mortality as a surrogate), variation by cancer site, systematic biases, or confounding. Of particular concern are potential time-related biases that may arise when evaluating metformin and cancer risk (100). A recent meta-analysis of observational and randomized studies attempted to account for major biases and confounders, still finding a significant, though attenuated, reduction in cancer incidence among studies without time-

related biases (n=8 studies; summary RR: 0.90; 95% CI: 0.89-0.91) and among studies adjusted for body mass index (n=11 studies; summary RR: 0.82; 95% CI: 0.70-0.96) (101). Observational studies published after these meta-analyses have either been consistent with reduced cancer risk (102, 103) or null (104-106). Overall, the literature suggests that metformin either reduces or has no effect on cancer risk, though very few studies have addressed metformin use in the non-diabetic population. Future clinical trials of metformin therapy in the general population should provide vital data on the potential use of metformin as a chemopreventive agent.

Metformin use may also influence disease progression after a cancer diagnosis. In observational studies, metformin has been associated with a decreased risk of disease recurrence, overall mortality, or cancer-specific mortality in patient cohorts of prostate cancer (107, 108), multiple myeloma (109), liver cancer (110), ovarian/endometrial cancer (110-112), bladder cancer (113, 114) and breast cancer (115, 116). Two additional studies of prostate cancer patients who underwent radical prostatectomy found no significant associations between metformin use and time to biochemical recurrence or longer-term outcomes (117, 118). Two additional studies of breast cancer patients were null for metformin use and overall or cancer-specific survival (119, 120). Numerous clinical trials of metformin as an adjuvant therapy to cancer treatment are underway as indicated on ClinicalTrials.gov. Combined with the observational data, these new clinical trials will shed light on the potential therapeutic role of metformin in cancer survivors.

In addition, a limited number of 'window of opportunity' (i.e. phase 0) trials have been conducted to evaluate metformin administration in the time window between cancer diagnosis and surgery. These studies show mixed results for tumor p-AMPKα at Thr172 expression before and after metformin use (ranging from 850-2250 mg/day): p-AMPKα protein expression was increased in one study of endometrial cancer patients (121), decreased in another study of endometrial cancer patients (122), and unchanged in two studies of endometrial (123) and prostate (124) cancer patients. Thus, a

direct link between short-term metformin use and AMPK activation in targeted tissue is unclear. Larger studies of longer duration and varying dosage of metformin use across various cancer types are needed to determine whether metformin acts through the AMPK pathway to influence tumor growth and progression.

Phenformin, a metformin analog, is also a potent indirect activator of AMPK and was administered as anti-diabetic medication starting in the mid-1900s. However, increased risk of lactic acidosis, often fatal, led to the withdrawal of phenformin by the US Food and Drug Administration in 1977 (125). Phenformin has a longer half-life and displays more potent anti-neoplastic activity compared to metformin in *in vitro* and *in vivo* pre-clinical studies (126). *In vitro* studies of the antitumorigenic effects of metformin are often at supra-physiological concentrations that may be unattainable in humans, thus phenformin may offer an alternative for chemoprevention or adjuvant therapy for cancer patients. Phenformin continues to be available in some parts of the world. In a recent cohort study of biguanide use and colorectal cancer risk in Denmark, phenformin comprised 0.5% of biguanide prescriptions (127). The investigators analyzed all biguanides as a group and found an increased risk of colorectal cancer among biguanide users compared to non-diabetics, and risk estimates were inconsistent when biguanide users were compared to diabetics on other oral anti-diabetic drugs. These results conflict with the much of the current literature suggesting a reduced risk or null association for biguanide treatment and colorectal cancer incidence (99).

More recently, salicylate, the metabolic derivative of aspirin, has been shown to directly activate AMPK (69). Aspirin has long been known to exhibit antineoplastic properties, though whether these properties are mediated by AMPK is unknown. Algra et al. summarized the results for any aspirin use and long-term cancer incidence, reporting summary RRs [95% CI] of 0.88 [0.84-0.92] among 150 case-control studies and 0.87 [0.83-0.91] among 45 cohort studies for risk of all cancer types, with the most consistent findings for reduced risk of colorectal cancer (128). Rothwell et al. summarized the results for

regular aspirin use and cancer incidence and mortality among randomized controlled trials for the primary prevention of cardiovascular disease, reporting summary RRs [95% CI] of 0.88 [0.80-0.98] for cancer risk among 6 trials and 0.85 [0.76-0.96] for cancer deaths among 34 trials (129). This group also found that aspirin use among patients with non-metastatic adenocarcinoma at diagnosis was associated with a reduced risk of subsequent metastasis (summary RR=0.45; 95% CI: 0.28-0.72) and cancer death (summary RR=0.50; 95% CI: 0.34-0.74) among 5 randomized trials of daily aspirin for the prevention of vascular events (130). Additional observational studies support an association between regular aspirin use after diagnosis and improved survival outcomes among breast (131, 132), colorectal [(133-137), reviewed in (138)], and prostate cancer (139, 140) patients, while other studies do not (141-144). Overall, the current evidence from long-term observational and randomized studies is strongly suggestive of a potential role for aspirin in the primary and secondary prevention of cancer.

In summary, observational and randomized studies suggest a potential benefit of AMPK-activating drugs for chemoprevention and/or improving cancer survival. These findings are in agreement with associations between AMPK activation levels in tumor tissue and more favorable clinicopathologic features and survival outcomes observed in several cancer types (Table 1). In future studies, it will be important to understand to what extent AMPK activation mediates the ability of these drugs to reduce cancer risk, and to define their action in the context of the metabolic status of the individual, concurrent medication use, and the natural history of cancer.

Conclusions

The duplicitous role of AMPK activation in cancer cells is context-specific and affects the outcome of AMPK modulation. More sophisticated genetic manipulation of AMPK is necessary to understand its biochemical and cell biology function in the different contexts. In addition, knowledge of long-term outcomes in healthy individuals and cancer patients in relation to AMPK status is necessary to inform the potential use of AMPK modulators in the clinical setting. Thus, the road towards a deeper

understanding of AMPK's role in cancer and its therapeutic exploitation is still under construction.

Figure Legends

Figure 1. Mechanisms of AMPK activation

AMPK functions as a metabolic sensor that is activated by metabolic stress induced by hypoxia, nutrient deprivation, and drugs/compounds [e.g. biguanides, 2-deoxyglucose (2-DG)], AMP mimetic, direct AMPK activators, or reactive oxygen species (ROS). For the full activity of the kinase, a phosphorylation at the residue Thr172 in the catalytic loop is required. The main upstream kinases are the Liver kinase B1 (LKB1), the Ca2+/calmodulin-dependent protein kinase kinase 2 (CaMKK2), and the transforming growth factor beta-activated kinase 1 (TAK1). Uncharacterized protein phosphates (PPs) can reverse this phosphorylation.

Figure 2. AMPK-mediated metabolic and signaling reprogramming

Once activated, AMPK switches off anabolic pathways while turning on catabolic pathways to restore energy homeostasis. Thus, AMPK controls pathways involved in metabolism, cell growth, and survival. Red lines indicate direct activation, whereas inhibition is depicted in blue. A question mark indicates that it is not yet certain that the protein is directly phosphorylated. Abbreviations: ACC1/ACC2, acetyl-CoA carboxylases 1/2; HMGR, HMG-CoA reductase; SREBP, sterol response element binding protein; CHREBP, carbohydrate response element binding protein; FAO, fatty acid oxidation; TIF-1A, transcription initiation factor-1A; mTORC1, mammalian target of rapamycin complex 1; TSC2, tuberous sclerosis complex 2, GLUT1/4, glucose transporter 1, 4; PFKFB2/3,6-phosphofructo-2-kinase/fructose-2, 6-bisphosphatases 2 and 3; TBC1D1, TBC1 domain protein-1; SIRT1, sirtuin 1; PGC-1α, PPARγ-coactivator-1α; ULK1, Unc51-like kinase-1, AMOTL1, angiomotin like 1; YAP, Yes-associated protein 1.

Figure 3. Main mechanisms through which AMPK can exert its double-faced role in cancer

AMPK activation triggers cellular processes that can both suppress and promote tumor development/progression by activating different downstream pathways in a context specific manner.

Abbreviations: mTORC1, mammalian target of rapamycin complex 1; HIF-1α, hypoxia-inducible factor 1-alpha; YAP, Yes-associated protein 1; Foxo3a, forkhead box O3; AR, androgen receptor; FAO, fatty acid oxidation; ACC2, acetyl-CoA carboxylases 2; NADPH, reduced form of nicotinamide adenine dinucleotide phosphate; ROS, reactive oxygen species; ULK1, Unc51-like kinase-1.

Figure 4. AMPK functions as "conditional" tumor suppressor and "contextual" tumor promoter.

The outcome of AMPK activation in cancer is affected by the genetic context, metabolic dependency of cancer cells, and the surrounding microenvironment. Differences in the intensity/duration of AMPK activation (e.g. physiological activation *vs.* drug-induced supra-physiological activation) as well as in the expression/activation of specific subunits of the heterotrimer contribute to the anti- *vs* pro-tumorigenic role of AMPK in different cancer types.

Figure 5. Mechanisms by which biguanides are therapeutically beneficial in LKB1-positive and negative tumors.

A. Metformin or phenformin activates AMPK in pre-neoplastic cells with functional LKB1/AMPK pathway, restraining their growth and proliferation and thus delaying the onset of tumorigenesis;

B. Cancer cells, in which the LKB1-AMPK pathway is not functional, cannot restore biguanides-induced energy stress and they are more sensitive to cell death (biguanide paradox).

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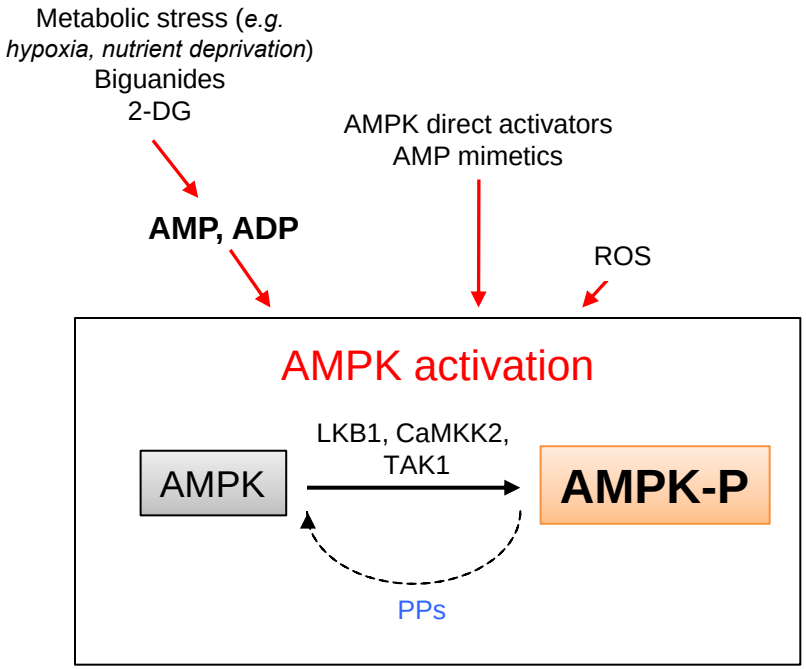

Figure 1

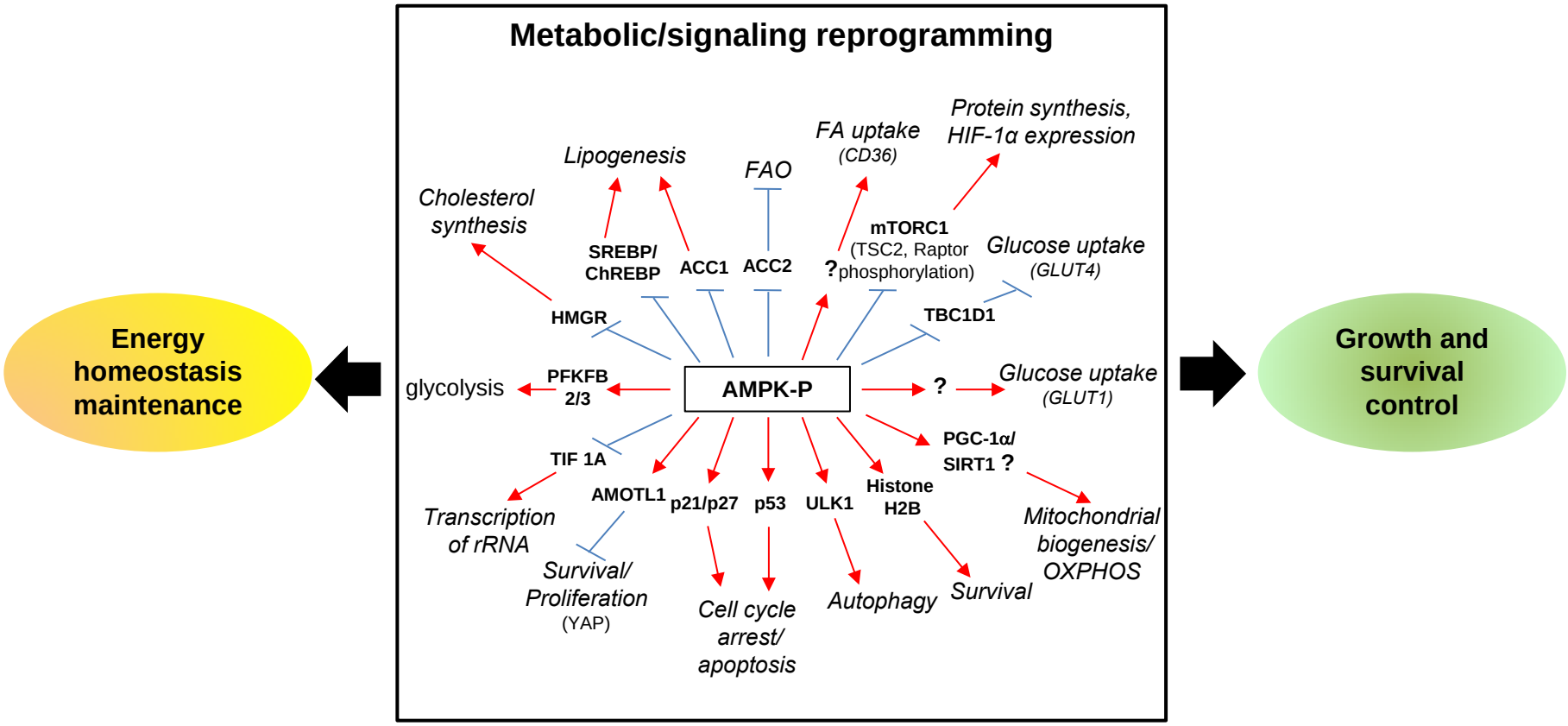

Figure 2

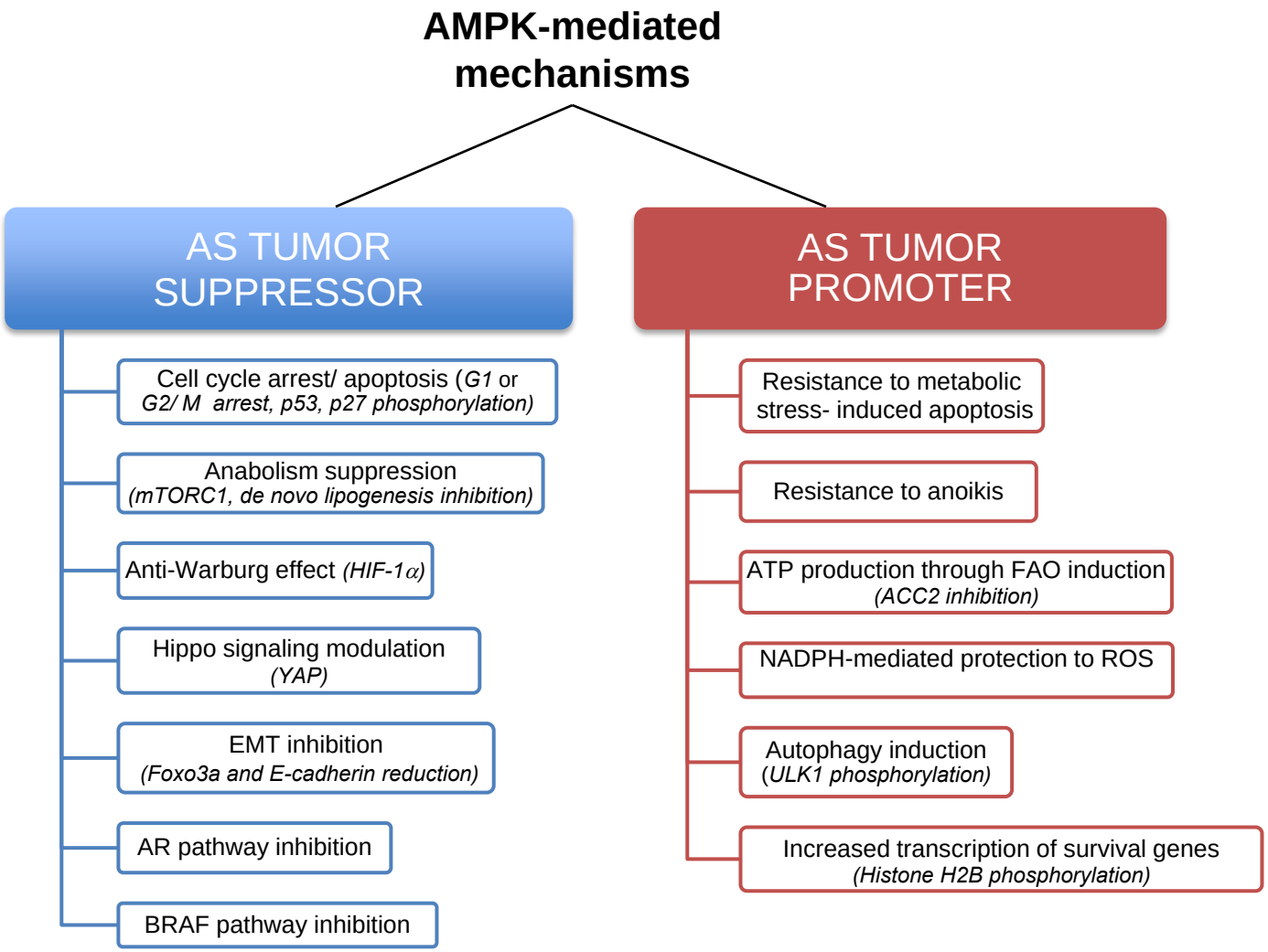

Figure 3

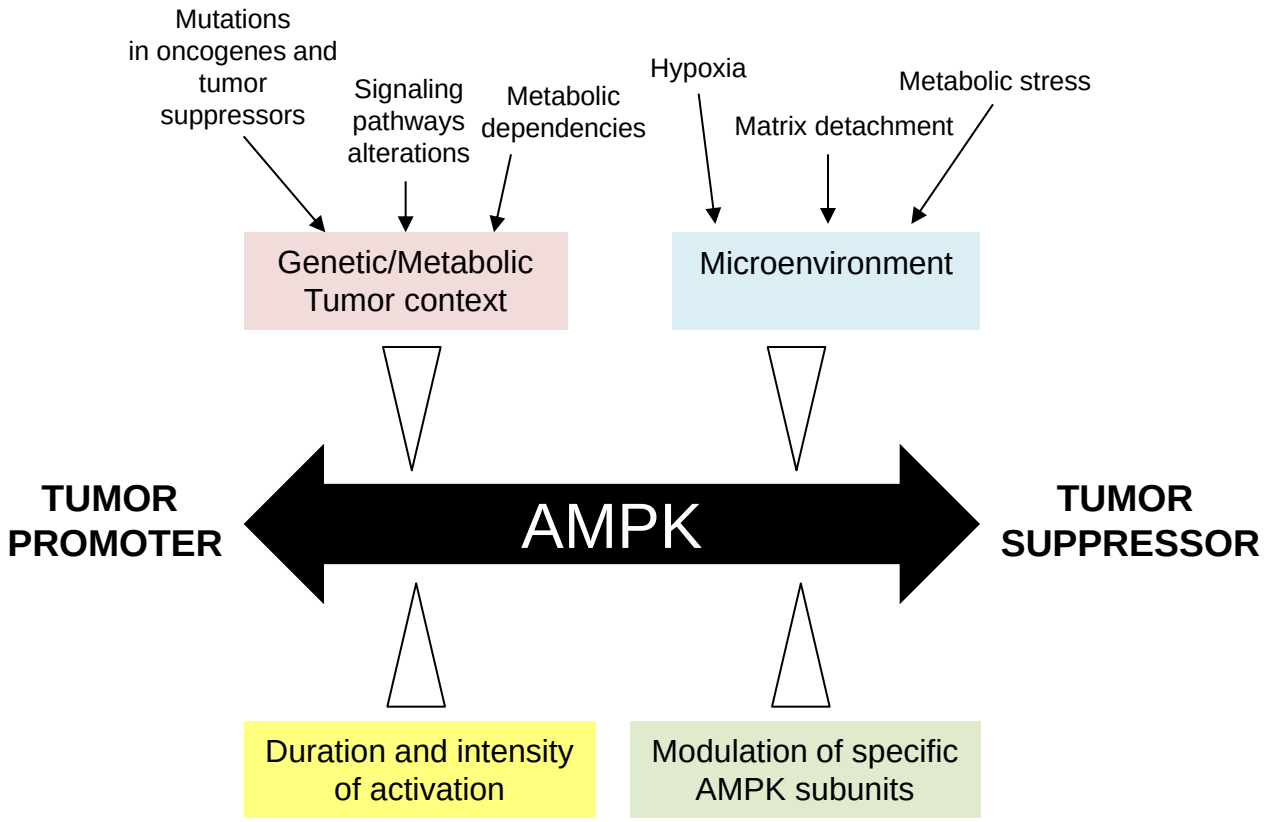

Figure 4

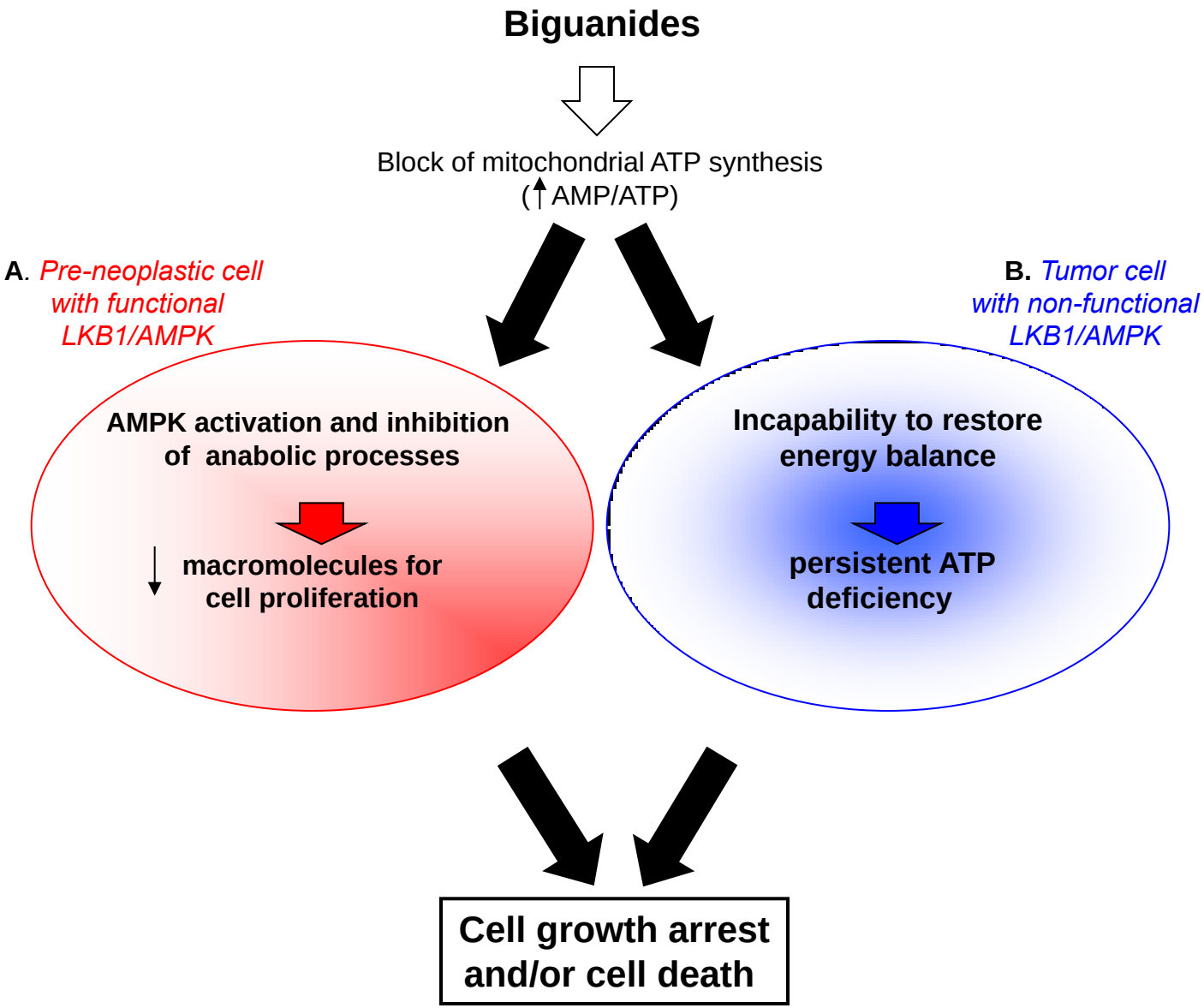

Figure 5

Table 1: Population-based studies of AMPK activation in tumor tissue, clinicopathologic features, and prognosis

Author, Year [ref.]	Cancer site	Country	Population	Age range, yrs	Time period of diagnosis	N cases	Median follow-up, yrs	Antibody used for AMPK activation; method	Main findings[1]		
									Overall, cancer-specific, & progression-free survival	Tumor grade & stage	Other clinicopathologic features
Park, 2009 [71]	Prostate	USA	Patients with paraffin-embedded arrayed prostate cancer specimens	NS	NS	244	NA	p-ACC (Ser79, Cell Signaling Technology); IHC		No association of p-ACC with Gleason grade (data not shown).	
Tennakoon, 2013 [72]	Prostate	USA	Patients with archival tissue collected from radical prostatectomy	NS	NS	61	NS	p-AMPKα (Thr172, Santa Cruz Biotechnology); IHC	Higher p-AMPK associated with biochemical recurrence (p=0.017).	No association of p-AMPK with Gleason score or disease stage at time of surgery.	
Choudhury, 2014 [63]	Prostate	UK	Patients with paraffin-embedded arrayed prostate cancer specimens	NS	NS	213	NA	p-AMPKα (Thr172); IHC		Higher p-AMPK associated with higher Gleason score (p=0.0251).	
Hadad, 2009 [73]	Breast	Scotland	1. Patients enrolled in Adjuvant Breast Cancer (ABC) clinical trial 2. Patients with primary, previously untreated breast cancer from Tayside University Hospitals	34-76 28-89	1992-2000 1997-2002	117 237	6.1 5.0	p-AMPKα (Thr172, Cell Signaling Technology); IHC	No association of p-AMPK with overall survival (data not shown).	Higher p-AMPK associated with lower histological grade (p=0.010 and 0.021 for cohorts 1 & 2, respectively).	Higher p-AMPK associated with fewer positive axillary nodes (p=0.021 and 0.087 for cohorts 1 & 2, respectively). No association with tumor size (data not shown).
Zhang, 2014 [74]	Breast	UK	1. Discovery cohort 2. Validation cohort Both comprised of stage I-III invasive breast cancers from patients treated by wide local excision and radiotherapy	31-70 18-72	1998-2006 1986-1998	166 609	9.0 11.2	p-AMPKα (Thr172, Cell Signaling Technology); IHC	No association of p-AMPK with overall survival (data not shown).	No association of p-AMPK with tumor grade or stage (data not shown).	No association of p-AMPK with tumor size or lymph node status (data not shown).
Su, 2014 [75]	Head and neck		Patients with surgically resected squamous cell carcinoma of the head and neck	30-89	1998-2010	118		p-AMPKα (Thr172, Cell Signaling Technology); IHC	Higher p-AMPK associated with improved overall survival in univariate (p=0.018), but not multivariate (p=0.188), analyses.	Higher p-AMPK associated with lower T stage (p=0.020). No association of p-AMPK with tumor differentiation (p=0.200).	No association of p-AMPK with surgical margin status (p=0.253) or lymph node status (p=0.369).
								p-ACC (Ser79, Cell Signaling	Higher p-ACC associated with worse overall		

								Technology); IHC	survival in univariate (p=0.021) and multivariate (p=0.018) analyses.	
Baba, 2010 [76]	Colorectal	USA	Incident cases in Nurses' Health Study and Health Professionals Follow-up Study	≤59 (20%); 60-69 (42%); ≥70 (38%)	1976-2004	718	10.8	p-AMPKα (Thr172, Cell Signaling Technology); IHC	No association of p-AMPK with cancer-specific survival (p=0.56) in all patients combined. Higher p-AMPK associated with improved cancer-specific survival among P-MAPK3/1 positive (p=0.0006), but not P-MAPK3/1 negative (p=0.45) patients.	Higher p-AMPK associated with lower tumor grade (p=0.0009). No association of p-AMPK with tumor stage (p=0.16).	No association of p-AMPK with tumor border (p=0.80).
Zulato, 2014 [77]	Colorectal	Italy	Patients with metastatic colorectal cancer treated with FOLFIRI-bevacizumab	28-74	2007-2011	48	2.0	p-AMPKα (Thr172, Cell Signaling Technology); IHC	Higher p-AMPK associated with improved overall survival (p=0.0002). No association with progression-free survival (p=0.231).		
								p-ACC (Ser79, Cell Signaling Technology); IHC	Higher p-ACC associated with improved overall survival (p=0.0007) and improved progression-free survival (p=0.011).		
Kang, 2012 [78]	Gastric	South Korea	Patients receiving a combination regimen of cisplatin and S-1	22-71	2006-2010	73	2.2	p-AMPKα (Cell Signaling Technology); IHC	Higher p-AMPK associated with worse relapse-free survival (p=0.022). No association with overall survival (p=0.102).	No association of p-AMPK and overall pathologic stage (p=0.955), T stage (p=0.708), N stage (p=0.807), or histology (p=0.142).	No association of p-AMPK with tumor size (p=0.600)
Kim, 2013 [79]	Gastric	South Korea	Patients who underwent surgical gastrectomy	24-85	2003-2006	621	Up to 10 yrs	p-AMPKα (Thr172, Cell Signaling Technology); IHC	Higher p-AMPK associated with improved overall survival (p=0.024) and disease-free survival (p=0.030).	Higher p-AMPK associated with lower tumor stage (p=0.000).	Higher p-AMPK associated with absence of lymph node metastasis (p=0.000).
Zheng, 2013 [80]	Liver	China	Patients who underwent radical resection	<50 (56%); ≥50 (44%)	2005-2009	273	2.7	p-AMPKα (Thr172, Cell Signaling Technology); IHC	Higher p-AMPK associated with improved overall survival (p=0.00029) and longer time to recurrence (p=0.00071).	Higher p-AMPK associated with lower pathologic tumor stage (0.00014) and lower Edmondson grade (0.00324).	Higher p-AMPK associated with complete tumor encapsulation (p=0.00235) and absence of distant metastasis (p=0.00281). No association of p-AMPK with tumor size (p=0.775) or

											multiplicity (p=0.0932).
William, 2011 [81]	Lung	USA	Patients who underwent surgical resection for non-small-cell lung cancer	32-90	1997-2005	463	4.1	p-AMPKα (Thr172, Cell Signaling Technology); IHC	Higher p-AMPK associated with improved overall survival (p=0.0009) and recurrence-free survival (p=0.0007) in all patients, and in patients with adenocarcinoma (p=0.0001 and 0.001, respectively). No association of p-AMPK with overall survival (p=0.35) or recurrence-free survival (p=0.11) in patients with squamous cell carcinoma.	No association of p-AMPK with overall pathologic stage (p=0.45), T stage (p=0.61), or N stage (p=0.66).	
Zupa, 2012 [82]	Lung	Italy	Patients who underwent surgical resection for non-small-cell lung cancer	43-83	1993-2005	47	NS	p-AMPK α1 (Ser485, Cell Signaling Technology); RPPA	Higher p-AMPK α1 at Ser485 (prevents AMPK activation) associated with worse overall survival (p=0.0041) among 28 pathologic stage N0 patients.		
								p-ACC (Ser79, Cell Signaling Technology); RPPA	Higher p-ACC associated with worse overall survival (p=0.0256) among 28 pathologic stage N0 patients.		
Nanjundan, 2010 [83]	Lung	USA	Patients who underwent surgical resection for non-small-cell lung cancer	48-81	NS	46	NS	p-AMPKα (Thr172, Cell Signaling Technology); RPPA	No association of p-AMPK with recurrence or survival (data not shown).		
								p-ACC (Ser79, Cell Signaling Technology); RPPA	Higher p-ACC associated with disease recurrence (p=0.010). No association with survival (data not shown).		
Li, 2014 [84]	Ovary	USA	Patients included on a commercially available ovarian cancer tissue array (OVC1021, Pantomics Inc.)	NS	NS	97	NA	p-AMPKα (Thr172, Cell Signaling Technology); IHC		Higher p-AMPK associated with lower tumor stage (data not shown).	
Tsavachidou-Fenner, 2010	Kidney	USA	Patients with metastatic renal cell	Median: 61	NS	37	NS	p-AMPKα (Thr172, Cell Signaling	Higher p-AMPK associated with improved overall		

[85]			carcinoma who underwent nephrectomy, post-treatment with bevacizumab–erlotinib or bevacizumab alone					Technology)[2]; RPPA	survival (p=0.0003).
Cancer Genome Atlas Research Network, 2013 [86]	Kidney	USA	Clear cell renal cell carcinoma patients included in the publicly available TCGA database	NS	NS	411	Up to 10 yrs	p-AMPKα (Thr172); RPPA	Higher p-AMPK associated with improved overall survival (p<0.0001).
								p-ACC (Ser79); RPPA	Higher p-ACC associated with worse overall survival (p<0.01).

Abbreviations: CI, confidence interval; HR, hazard ratio; IHC, immunohistochemistry; NA, not applicable; NS, not specified; p-ACC, phosphorylated *acetyl-CoA carboxylase;* p-AMPK, phosphorylated AMP-activated protein kinase; *P-MAPK3/1,* extracellular signal-regulated kinase (ERK)1/2; RPPA, reverse-phase protein array; TCGA, The Cancer Genome Atlas

[1] Color code: Green: improved survival or favorable clinical features associated with AMPK activation; Red: worse survival or unfavorable clinical features associated with AMPK activation; Gray: null results

[2] Personal communication

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Molecular Cancer Research

Dissecting the Dual Role of AMPK in Cancer: from Experimental to Human Studies

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